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(54) Title: METHODS FOR THE DETECTION, TREATMENT, AND PREVENTION OF NEURODEGENERATION (57) Abstract In general, the invention provides methods for identifying genes involved in neurodegeneration and therapeutics for treating animals with a neurodegenerative disease. Methods and kits for the detection of compounds which enhance neuroprotection and diagnostic kits for the detection of neurodegenerative diseases are also a part of the invention.		

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METHODS FOR THE DETECTION, TREATMENT,
AND PREVENTION OF NEURODEGENERATION

Statement as to Federally Sponsored Research

This invention was made in part with support from the Federal government through NIH Grant No. 1RO1NS32196-04. The Federal government has certain rights in the invention.

Background of the Invention

The invention relates to methods and reagents for diagnosing, treating, and preventing neurodegeneration.

Loss of neurons by a degenerative process is a major pathological feature of many human neurological disorders. Neuronal cell death can occur as a result of a variety of conditions including traumatic injury, ischemia, neurodegenerative diseases (e.g., Parkinson's disease, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), stroke, or trauma), or as a normal part of tissue development and maintenance. Several inherited disorders produce late onset neuron loss, each of which is highly specific for particular neural cell types. Nine genes have been cloned that are associated with susceptibility to these various neurological disorders (e.g., Huntington's disease, ataxin, and ALS); however, only in the case of Kennedy's syndrome is the biochemical function of the affected gene, the androgen receptor, understood (La Spada et al., Nature 352: 77-79, 1991). Epileptic seizures and stroke also produce neurodegeneration in humans and rodents.

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Summary of the Invention

In general, the invention features methods for the detection, treatment, and prevention of disorders involving neurodegeneration.

In a first aspect, the invention features a method for identifying a compound to treat or prevent the onset of a neurodegenerative disorder. The method involves contacting a cell that includes a reporter gene operably linked to a cAMP regulatory gene or promoter with a candidate compound and measuring the expression of the reporter gene, where a change in reporter gene expression in response to the candidate compound identifies a compound that is useful to treat or prevent the onset of a neurodegenerative disorder.

In various preferred embodiments of the first aspect of the invention, the cAMP regulatory gene may be an *acy-1* gene, an *eat-4* gene, an *unc-36* gene, or a glutamate receptor-encoding gene. In another preferred embodiment, the change in reporter gene expression is a decrease in expression.

In a second aspect, the invention features a cell for identifying a compound to treat or prevent the onset of a neurodegenerative disorder that includes a reporter gene operably linked to a cAMP regulatory gene or promoter.

In various embodiments of the above aspects, the cell is present in an animal, which may be a nematode (e.g., *C. elegans*) or a mammal (e.g., a rodent).

In a third aspect, the invention features a method for treating or preventing the onset of a neurodegenerative disorder in a mammal that includes administering to the mammal a therapeutically effective amount of a compound that decreases a neuronal cAMP level. In a preferred embodiment of this aspect of the invention, the mammal is a human.

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In a fourth aspect, the invention features a method for identifying a mammal (for example, a human) having or likely to develop a neurodegenerative disorder which includes determining whether the mammal has an increased level of cellular cAMP in a neuron, where an increased level indicates that the mammal has or is likely to develop a neurodegenerative disorder.

In a fifth aspect, the invention features a method for identifying a mammal (for example, a human) having or likely to develop a neurodegenerative disorder which involves determining whether the mammal has a mutation in a cAMP regulatory gene, where the presence of a mutation is an indication that the mammal has or is likely to develop a neurodegenerative disorder. In various preferred embodiments of this aspect, the mutation is in an adenylyl cyclase gene (e.g., the *acy-1* gene), or in an *unc-36* or *eat-4* gene. In other preferred embodiments, the mutation is in a gene encoding a $G\alpha_s$ subunit; and the mutation results in an increase in a neuronal cAMP level.

In a preferred embodiment of various aspects of the invention, the neurodegenerative disorder is Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer's disease, stroke, or epilepsy.

In a sixth aspect, the invention features a method for identifying a gene involved in neurodegeneration that involves providing a nematode (for example, *C. elegans*) that includes an expression construct that includes a promoter derived from a cAMP regulatory gene operably linked to a reporter gene, isolating a mutant of the nematode exhibiting an altered level of reporter gene expression, and identifying the gene comprising the mutation, wherein the gene is involved in neurodegeneration.

In a seventh aspect, the invention features a method for identifying a

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gene involved in neurodegeneration that involves providing a nematode (for example, *C. elegans*) that includes a glutamate receptor (GluR) promoter operably linked to a gene encoding a GTP-ase defective $G\alpha_s$ subunit, isolating a mutant of the nematode exhibiting a decreased level of paralysis and neurodegeneration, and identifying the gene that includes the mutation, wherein the gene is involved in neurodegeneration.

In an eighth aspect, the invention features a mammalian (for example, a human) EAT-4 polypeptide, and a vector and cell containing the nucleic acid.

In a ninth aspect, the invention provides a method for identifying a gene involved in neurodegeneration involving the steps of a) providing a cell that includes a cAMP regulatory gene promoter operably linked to a reporter gene; b) introducing into the cell a candidate gene capable of expressing a candidate protein; and c) measuring reporter gene expression in the cell, where an increase in reporter gene expression in the presence of the candidate protein indicates that the candidate gene is involved in neurodegeneration.

In preferred embodiments, the cell is yeast; and the cAMP regulatory gene is an *acy-1* gene, an *eat-4* gene, an *unc-36* gene, or a glutamate receptor-encoding gene.

In a tenth aspect, the invention features the use of a therapeutically effective amount of a compound that decreases a neuronal cAMP level in the manufacture of a medicament for treating or preventing the onset of a neurodegenerative disorder in a mammal (*e.g.*, a human).

As used herein, by "protein" or "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (*e.g.*, glycosylation or phosphorylation).

By "neurodegenerative disorder" is meant a disorder which is

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characterized by the death or loss of function of neuronal cells, also known as neurons. Neuronal death or loss of function can be associated with a number of diseases and syndromes including, without limitation, stroke, epilepsy, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, multiple sclerosis, and Alzheimer's disease.

By "G α_s -induced toxicity" is meant the neurodegeneration resulting from expression of the GTP-ase defective G α_s protein.

By "reporter gene" is meant any gene which encodes a product whose expression is detectable. A reporter gene product may have one of the following attributes, without restriction: fluorescence (e.g., green fluorescent protein), enzymatic activity (e.g., lacZ), toxicity (e.g., HER-1), or an ability to be specifically bound by a second molecule (e.g., biotin or a detectably labelled antibody).

By "cAMP regulatory gene" is meant any gene whose product regulates or is regulated by cAMP. Exemplary gene products include ACY-1, UNC-36, and EAT-4. Other preferred cAMP regulatory gene products include the ionotropic (cation) glutamate receptors (iGluRs), the Cl⁻ ionotropic glutamate receptors (GluCls), and the metabotropic glutamate receptors (mGluRs).

By "operably linked" is meant that a gene and a regulatory sequence are connected in such a way as to permit expression of the gene product under the control of the regulatory sequence.

By "purified nucleic acid" is meant DNA that is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autosomally replicating plasmid or virus; or into the genomic DNA or a

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prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

5 By a "transgene" is meant a nucleic acid sequence which is inserted by artifice into a cell and becomes a part of the genome of that cell and its progeny. Such a transgene may be partly or entirely heterologous to the cell.

By "mammalian *eat-4* polypeptide or mammalian EAT-4" is meant an amino acid sequence derived from a mammalian cell which shares at least
10 50%, preferably 70%, more preferably 80%, and most preferably 90% amino acid sequence identity with a *C. elegans eat-4* amino acid sequence (SEQ ID NO: 1). Preferably, such a polypeptide is capable of at least partially complementing a *C. elegans eat-4* mutation.

By "*acy-1* polypeptide or ACY-1" is meant an amino acid sequence
15 which is substantially identical to the amino acid sequence provided in Fig. 5 (SEQ ID NO: 2).

By "substantially identical" is meant an amino acid sequence or nucleic acid sequence which shares identity with another of the same class. Preferably, such a sequence is at least 85%, more preferably 90%, and most
20 preferably 95% identical to the sequence described in the references provided herein. For polypeptides, the length of comparison sequences will generally be at least 15 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably at least 35 amino acids. For nucleic acids, the length of comparison sequences will be at least 45 nucleotides,
25 preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably at least 105 nucleotides. Identity is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the

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Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of identity to various substitutions, deletions, substitutions, and other modifications.

5 Other features and advantages of the invention will be apparent from the following detailed description thereof, and from the claims.

Brief Description of the Drawings

Figs. 1A and 1B are photographs of neuronal cells from young
10 *Caenorhabditis elegans* larvae co-expressing green fluorescent protein (GFP) with GTP-ase defective rat $G\alpha_s$ as seen morphologically (Fig. 1A), as well as in bright field optics (Fig. 1B).

Fig. 2 is a table listing the extent of swelling and cytotoxicity of PVC neurons resulting from the expression of the $\alpha_s(gf)$ transgene in various genetic
15 backgrounds. Statistical differences between genotypes were determined by the method of attributable risk described in J. Devore, Probability and statistics for engineering and the sciences (Brooks/Cole, Belmont, ed. second, 1987). Multiple comparisons were compensated for by setting $p < 0.005$ as the threshold for significance.

20 Fig. 3 is an amino acid sequence of the EAT-4 protein (SEQ ID NO: 1).

Fig. 4 is a schematic diagram showing the genetic and physical map position of the *acy-1* gene on the F17C8 cosmid.

Fig. 5 is a set of schematic diagrams of the predicted structures of the
25 *acy-1* gene and the GFP fusion protein (KP#107). Positions of the *acy-1* mutations *nu327*, *nu343*, and *nu329* are indicated.

Fig. 6 is an amino acid sequence of the ACY-1 protein (SEQ ID NO:

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2). The ACY-1 sequence (top) is shown aligned with the mouse adenylyl cyclase type 9 amino acid sequence (bottom) (SEQ ID NO: 3). Underlined sequences indicate predicted transmembrane domains. Positions of the *acy-1* mutations *nu327*, *nu343*, and *nu329* are indicated.

5 Figs. 7A and 7B are photographs of GFP-expressing PVC neurons in adult $\alpha_s(gf)$ (Fig. 7A) and adult $\alpha_s(gf);acy-1(nu343)$ (Fig. 7B) *C. elegans*.

Figs. 8A and 8B are photographs illustrating KP#107 *acy-1::gfp* fusion gene expression in neurons (Fig. 8A) and muscle (Fig. 8B).

10 Figs. 9A and 9B are photographs of PVC neurons from *unc-18* L1 larvae as seen with bright field (Fig. 9A) and fluorescence (Fig. 9B) optics.

Figs. 10A and 10B are photographs of PVC neurons from *unc-18* adults as seen with bright field (Fig. 10A) and fluorescence (Fig. 10B) optics.

Detailed Description of the Invention

15 The invention described herein is based upon genetic studies of the nematode, *Caenorhabditis elegans*. Constitutive activation of the GTP-binding protein $G\alpha_s$ was found to induce neurodegeneration. A screen for mutations that blocked $G\alpha_s$ -induced killing identified a gene, *acy-1*, which encodes a protein that is highly similar (40% identical) to mammalian adenylyl cyclases,
20 indicating that $G\alpha_s$ -induced neurotoxicity is likely mediated by changes in cyclic adenosine monophosphate (cAMP) levels. This discovery enables methods and reagents for diagnosing and treating neurodegeneration.

$G\alpha_s$ -induced neurotoxicity

25 Although neurodegeneration is a major feature in a variety of human neurological disorders, relatively little is known about the signal transduction pathways that lead to neurotoxicity, nor how these pathways could be

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manipulated to protect against neuron loss in these diseases. Two critical questions in the pathogenesis of human neurodegenerative disorders are (1) what factors predispose particular neurons to undergo degeneration and (2) what is the biochemical mechanism of degeneration. A genetic model for excitotoxicity in the nematode *Caenorhabditis elegans* was developed to address these questions.

In particular, a rat cDNA encoding a GTPase-defective (Q227L) $G\alpha_s$ subunit, hereafter referred to as $\alpha_s(gf)$, was expressed in *C. elegans* neurons using the *glr-1* glutamate receptor (GluR) promoter. The expression vector, KP#20, was constructed by inserting into a derivative of the *C. elegans glr-1* expression vector CX#1 (as described in Chalfie et al., Science 263: 802-805, 1994), a 1.5 kb *NcoI-XhoI* fragment encoding a GTPase defective (Q227L, KP#20) mutant rat $G\alpha_s$ cDNA. *C. elegans* transgenic for $\alpha_s(gf)$ were prepared by microinjecting the KP#20 expression construct together with a *glr-1::gfp* plasmid (the KP#6 vector) using *lin-15* (Huang et al., Mol. Biol. Cell. 5, 395-412, 1994) as a transformation marker. A stable line carrying *glr-1* expression constructs for both GFP and the GTPase defective $G\alpha_s(nuls5)$ was isolated following 3500 rads of γ -irradiation. The *glr-1* promoter was chosen because it is highly expressed, and because *glr-1*-expressing cells control locomotion, an easily assayed behavior. The *glr-1* promoter is expressed in 17 classes of neurons, including the interneurons (AVB, PVD, AVA, and AVD) required for locomotion. The *glr-1* expressing neurons are as follows: AVG, AVJ, DVC, PVC, PVQ, RIG, RIS, RMD, RMEL/R, SMD, URY, as well as the six ASH synaptic targets AIB, AVA, AVB, AVD, AVE, and RIM (Hart et al., Nature 378: 82-85, 1995; Maricq et al., Nature 378: 78-81, 1995).

Since $G\alpha_s$ was co-expressed with the green fluorescent protein (GFP) of *Aequorea* (Chalfie et al., *supra*), examination of the morphology of

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$G\alpha_s$ -expressing cells was possible. Transgenic *glr-1::\alpha_s(gf)* animals were found to be paralyzed. As shown in Figs. 1A and 1B, a subset of the $G\alpha_s$ -expressing neurons in young larvae swelled to several times their normal diameter. The swelling was apparent by the morphology of GFP expressing cells (Fig. 1A) and by their appearance in bright field optics (Fig. 1B) as enlarged, apparently vacuolated cells often with an intact nucleus. The interneurons AVE and AVD were swollen compared to neighboring unaffected cells which have been marked in Figs. 1A and 1B with asterisks. 88% of the PVC neurons swelled, 5% of RIG neurons swelled, and none of the URY cells swelled in first stage (L1) *glr-1::\alpha_s(gf)* larvae. The neurotoxicity occurred in two phases; subsequent to swelling, the swollen cells eventually disappeared, presumably because the cells had died. In *glr-1::\alpha_s(gf)* animals, 89% of the PVC neurons degenerated, as summarized in the table in Fig. 2. Other *glr-1* expressing cells degenerated at lower frequencies, including AVA, AVD, AVE, AVG, PVQ, RIG, and SMD. Expression of a constitutively active rat $G\alpha_s$ cDNA was found to cause neurotoxicity in *C. elegans*. Characterization of the neurodegenerative phenotype in the resulting *glr-1::\alpha_s(gf)* was made as follows: Swollen or missing cells were identified by examining the morphology of GFP-expressing cells. $G\alpha_s$ -induced neurotoxicity in various genetic backgrounds was quantitated as the number of swollen PVC neurons in L1 larvae, and the percentage of PVC neurons that were missing or swollen in adults hermaphrodites. These results suggested that exaggerated $G\alpha_s$ signaling killed neurons.

The phenotype of $G\alpha_s$ -induced neurotoxicity was identical to the neurotoxicity due to excessive signaling by the excitatory neurotransmitter glutamate, which has been termed excitotoxicity. Excitotoxic neuron loss occurs in two phases. First, acute neuron loss is associated with swelling of

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cell bodies and is dependent on extracellular ionic conditions. Cell swelling is the consequence of depolarization of membrane potential by excitotoxic agonists, which leads to the influx of Na^+ and Cl^- ions, and water (Olney, Adv. Exp. Med. Biol. 203: 631-645, 1986; Choi, J. Neurosci. 7: 369-379, 1987; Choi, Neuron 1: 623-634, 1988). Second, delayed neuron loss in excitotoxicity is not dependent on the extracellular ionic conditions, but is correlated with elevations of intracellular Ca^{2+} and chronic activation of immediate early genes (e.g., fos and jun) (Smeyne et al., Nature 363: 166-169, 1993). Hence, $\text{G}\alpha_s$ -induced neurotoxicity is most likely excitotoxicity.

Neurons differed greatly in their susceptibility to $\text{G}\alpha_s$ -induced toxicity

The *mec-7* gene product, MEC-7 tubulin, is abundantly expressed in 5 neurons, called touch cells, that sense light touch to the worm's body (Savage et al., Genes Dev. 3: 870-81, 1989; Hamelin et al., EMBO 11: 2885-2893, 1992; Mitani et al., Development 119: 773-783, 1993). To further investigate the specificity of $\text{G}\alpha_s$ -induced toxicity, $\alpha_s(gf)$ was expressed in *C. elegans* utilizing the *mec-7* promoter. The *mec-7::\alpha_s(gf)* expression plasmid (KP#7) was constructed by ligating the 1.5 kb *NcoI-XhoI* $\text{G}\alpha_s(\text{Q227L})$ into the *mec-7* expression vector pPD52.102. *C. elegans* transgenic for the *mec-7::\alpha_s(gf)* expression plasmid were prepared by microinjecting the KP#7 expression construct together with a *mec7::gfp* plasmid using *lin-15* (Huang et al., *supra*) as a transformation marker. A stable line carrying *mec-7* expression constructs for both GFP and the GTPase defective $\text{G}\alpha_s(nuIs5)$ was isolated following γ -irradiation.

C. elegans expressing the *mec-7::\alpha_s(gf)* transgene were found to be indistinguishable from wild type animals, having no obvious defect in touch sensitivity nor in the morphology of the touch cells. Hence, the effects of $\text{G}\alpha_s$

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on neural activity and on neurotoxicity were cell type specific.

Mutations that blocked $G\alpha_s$ -induced neurotoxicity

Both the *glr-1* and the *mec-7* expression constructs supported the notion that the effects of $G\alpha_s$ on neural activity and on neurotoxicity were cell type specific. Since the *mec-7* promoter is very highly expressed in the touch neurons (Savage et al., *supra*; Hamelin et al., *supra*; Mitani et al., *supra*), the results also suggested that the cell type specificity could not be overcome by high levels of $G\alpha_s$ expression. To identify the targets of $G\alpha_s$, mutations that block $G\alpha_s$ -induced paralysis and neurotoxicity were isolated by identifying mutations isolated from the F2 self-progeny of EMS mutagenized (5 μ l/ml) hermaphrodites that restored normal locomotion rates to $\alpha_s(gf)$ homozygotes. Candidate suppressor mutants (7500 haploid genomes) were subsequently screened for reduction of $G\alpha_s$ -induced swelling in L1 larvae which led to the isolation of 3 semidominant mutations which blocked $G\alpha_s$ -induced paralysis and neurotoxicity

Mutations in *acy-1* blocked $G\alpha_s$ -induced neurotoxicity

In two factor mapping experiments, the three mutations that blocked $G\alpha_s$ -induced neurotoxicity were all found to be linked to *dpy-17*. Three factor mapping placed these mutations between *emb-5* and *dpy-17*: (*nu327 dpy-17*) 37/37 *unc-32*; (*nu329 dpy-17*) 16/16 *unc-32*; (*nu343 dpy-17*) 4/4 *unc-32*; *unc-79* (6/14) MJ#NEC2 (5/14) *nu329* (3/14) *dpy-17*; *emb-5* (1/16) *nu327* (15/16) *dpy-17*. As illustrated in the schematic diagram of Fig. 4, two of the three mutations were mapped to a 1.5 cM genetic interval between MJ#NEC2 and *dpy-17* on the F17C8 cosmid. The cosmid was then microinjected into *acy-1(nu327)*; *nuIs5* animals, and transgenic lines were isolated using

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goa-1::gfp (KP#13) (Segalat, et al., Science 267, 1648-1651, 1995) as a transformation marker. Four independent lines carrying a cosmid from this interval (F17C8) were obtained, two of which corrected the mutant phenotype of *acy-1(nu327)* animals, i.e., they had increased degeneration of the PVC neurons. This is shown on Table 1.

Table 1

**Transgenes containing the F17C8 cosmid
rescue the *acy-1(nu327)* mutant phenotype**

genotype	% PVC degeneration
<i>acy-1(nu327);$\alpha_s(gf)$</i>	12
<i>acy-1(nu327);$\alpha_s(gf)$; nuEX(F17C8)</i>	75
<i>$\alpha_s(gf)$</i>	88

In addition, Fig. 5 shows that all three alleles corresponded to mutations in the predicted exons of the gene F17C8.1, one of two predicted adenylyl cyclase genes in the *C. elegans* genome database. This adenylyl cyclase gene has been named *acy-1*. Furthermore, Fig. 6 shows the results of a Genbank database scan for sequences related to *acy-1* (SEQ ID NO: 2). The amino acid sequence of ACY-1 was found to be 40% identical at the amino acid level to mouse adenylyl cyclase type 9. It is unclear why the *acy-1* mutations were partially dominant. Analysis of the molecular nature of the mutations suggested that they were simple loss of function mutations. For example, *nu329* and *nu343* were predicted to disrupt pre-mRNA splicing. Indeed, as is shown in Figs. 7A and 7B, the GFP-expressing PVC neurons which were typically missing in *$\alpha_s(gf)$* adult transgenic worms (Fig. 7A) were present in *$\alpha_s(gf);acy-1(nu343)$* (Fig. 7B). Thus, it is possible that *$\alpha_s(gf)$* animals

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were highly sensitive to changes in cAMP levels. Overall, the results suggested that $G\alpha_s$ neurotoxicity was mediated by changes in intracellular cAMP.

Physiological function of ACY-1

5 To determine the physiological function of ACY-1, an analysis of *acy-1* expression was carried out. A deleted derivative (KP#106) of the cosmid F17C8 was isolated by digesting with *Afl*III and re-ligating. KP#106 contained the entire 8.35 kb *acy-1* genomic region together with the 5.2 kb 5' and 4.9 kb 3' flanking sequences. An *acy-1::gfp* expression vector (KP#107) was
10 constructed by PCR amplification of a 1.7 kb fragment containing the GFP coding region and the *unc-54* transcription terminator from pPD95.75, followed by ligation of this fragment into the unique *Asp*718 site in KP#106, creating a fusion protein containing the first 6 exons of *acy-1* fused to GFP. The
15 ACY-1::GFP fusion protein contained 6 predicted transmembrane domains of ACY-1, and was therefore membrane localized. Transgenic animals carrying KP#107 were isolated by microinjection using *lin-15* (Huang et al., *supra*) as a transformation marker. Expressing cells were identified based on their morphology and nuclear positions.

 The expression pattern of *acy-1* was determined by analyzing the
20 GFP reporter construct. As is shown in Figs. 8A and 8B, the *acy-1::gfp* fusion protein was expressed in virtually all neurons (Fig. 8A) and body muscles (Fig. 8B). In Fig. 8A, ACY-1 expression in the two ventral rows of body muscles (arrows) and in the ventral cord neurons and neuropile (lines) is shown. In Fig. 8B, expression of ACY-1 in the vulva muscles (arrow heads) is shown. Nearly
25 all of the 302 neurons in adult *C. elegans* appeared to express ACY-1. Cell bodies were identified based upon the bright fluorescence in the intracellular membranes (which are presumably the endoplasmic reticulum of Golgi

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apparatus). ACY-1 did not appear to be expressed in non-neural tissues or in the pharynx. These results indicated that the ACY-1 adenylyl cyclase is likely to participate in many neural signaling pathways. Therefore, we expected that *acy-1* mutants would have defects in behavior or development. Consistent with this notion is that mutations which inactivated the *C. elegans* $G\alpha_s$ subunit (GSA-1) were found to be homozygous lethal. Surprisingly, we observed that *acy-1* homozygotes were nearly indistinguishable from wild type animals. This result suggested that the essential function of GSA-1 was mediated by some other adenylyl cyclase. Alternatively, *acy-1* and other adenylyl cyclases could act redundantly in the essential GSA-1 pathways.

Activated $G\alpha_s$ induced neurotoxicity by excitotoxicity

Several previously identified genes were considered good candidates for mediating the toxic effects of $G\alpha_s$. Two cyclic nucleotide gated ion channel (CNGC) subunit genes *tax-2* and *tax-4* (Coburn and Bargmann, Neuron 17: 695-706, 1996; Komatsu et al., Neuron 17: 707-718, 1996) are not expressed in *glr-1* expressing cells and hence are unlikely targets. The *mec-6*, *unc-8*, and *deg-1* genes have been previously implicated in neurodegeneration (Chalfie and Wolinsky, Nature 345: 410-416 (1990); Driscoll and Chalfie, Nature 349: 588-593, 1991; Shreffler et al., Genetics 139: 1261-1272, 1995; Tavernarakis et al., Neuron 18: 107-119, 1997), and the DEG-1 and UNC-8 proteins are similar to mammalian epithelial sodium channel subunits (ENaC), which are potently activated by cAMP-dependent protein kinase (PKA) (Sariban-Sohraby et al., J. Biol. Chem. 263: 13875-13879, 1988; Oh et al., Am. J. Physiol. 265: C85-C91, 1993; Bubien et al., J. Biol. Chem. 269: 17780-17783, 1994). The *unc-2*, *unc-36*, and *egl-19* genes encode subunits of voltage-dependent Ca^{2+} -channels (Schafer and Kenyon, Nature 375: 73-78, 1995) which are likely to be

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regulated by PKA (Curtis and Catterall, Proc. Natl. Acad. Sci. USA 82: 2528-2532, 1985) and have also been implicated in neurodegeneration. The *glr-1* gene encodes an ionotropic GluR (Hart et al., *supra*; Maricq et al., *supra*). GluRs have been implicated in neurotoxicity in mammals (Olney, Adv. Exp. Med. Biol. 203: 631-645, 1986; Choi, J., Neurosci. 7: 369-379, 1987; and Choi, Neuron 1: 623-634, 1988), and PKA augments the response of mammalian neurons to glutamatergic agonists (Greengard et al., Science 253: 1135-1138, 1991).

To examine the above genes for a possible role in $G\alpha_s$ induced toxicity, the neurodegenerative phenotype was characterized as described above. As shown in Fig. 2, of the candidate genes, only the *unc-36* mutation significantly reduced $G\alpha_s$ -induced cytotoxicity. Interestingly, the *unc-36* mutation had no effect on cell swelling. Since UNC-36 Ca^{2+} channels were required for cytotoxicity, these results suggested that $G\alpha_s$ cytotoxicity was mediated in part by either Ca^{2+} influx or depolarization of the affected cells. All other candidate genes had no effect on either neuron swelling or deaths in *glr-1::\alpha_s(gf)* animals. Our results do not exclude the possibility that these other candidate PKA targets also play a role in $G\alpha_s$ -induced toxicity. For example, more than one type of channel may be capable of mediating the toxic effects of $G\alpha_s$, in which case neurotoxicity would be prevented only in multiply mutant animals.

The *glr-1* mutation was unlikely to completely abolish glutamate signaling *in vivo*

Given its role in excitotoxicity in mammals, the requirement of endogenous glutamate signaling for $G\alpha_s$ neurotoxicity was tested. Although the *glr-1* mutation was not neuroprotective, it was possible that cAMP toxicity

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was mediated by exaggerated responses to endogenous glutamate. The *C. elegans* genome sequence (currently ~70% complete) predicted six additional ionotropic GluR subunits; therefore, the *glr-1* mutation was unlikely to completely abolish glutamate signaling *in vivo*.

5

Eat-4 mutant alleles eliminated ASH-mediated touch sensitivity

Prior work had shown that ASH sensory neurons mediated an aversive response to three distinct stimuli (nose touch, osmotic shock, and volatile repellents), and that the ASH-mediated touch response required functional GLR-1 glutamate receptors in synaptic targets of ASH (Hart et al., *supra*; Maricq et al., *supra*; Kaplan and Horovitz, Proc. Natl. Acad. Sci. (USA) 90: 2227-2231, 1993; Troemel et al., Cell 83: 207-218, 1995). Hence, genes required for ASH sensory responses were tested for their ability to perturb glutamate signaling.

15 We screened 11,000 mutagenized haploid genomes for animals that failed to respond to nose touch. Mutants isolated were subjected to a series of secondary screens, including dye-filling of the amphid sensory neurons, and responsiveness to osmotic shock and volatile repellents. Seven alleles of *eat-4* were isolated in this screen, all of which were normal for dye-filling but were defective for all three ASH sensory behaviors. The amino acid sequence of the EAT-4 is shown on Fig. 3. ASH-mediated sensory responses to nose touch, osmotic shock, and volatile repellents were compared in wild type and *eat-4*, as has been previously described (Hart et al., *supra*; Maricq et al., *supra*; Kaplan and Horovitz, *supra*; Troemel et al., *supra*). Briefly, for nose touch, animals were tested 10 times each with a positive response being scored when animals either halted forward movement or initiated backward movement following the stimulus. For osmotic avoidance, 50-60 animals were placed in 1 cm rings

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formed with 8 M glycerol, and the number of animals that escaped the ring after 9 minutes were counted. For volatile avoidance, an eyelash was dipped in 1-octanol and held near an animal's nose, and responses were quantitated by recording the length of time that elapsed before the animals reversed

5 locomotion.

All seven *eat-4* strains isolated had similar behavioral defects. In particular, as is portrayed in Table 2, *eat-4* strains had severe defects in the ASH-mediated touch, osmosensory, and volatile repellent responses.

10

Table 2

Role of *eat-4* in ASH sensory responses

Genotype:	Nose Touch (% Respond)	Osmotic Avoidance (% Escape)	Volatile Avoidance (seconds)
wild type	86 +/- 3	2 +/- 1	2.9 +/- 0.9
<i>eat-4(ky5)</i>	1 +/- 1	75 +/- 6	9.9 +/- 1.6
15 <i>eat-4(n2474)</i>	2 +/- 1	54 +/- 6	9.6 +/- 1.5

Errors indicate standard error of the mean in all cases. The number of animals and trials for each genotype were as follows: for nose touch, 10 animals and 100 trials; for osmotic avoidance, 60 animals and 5 trials; and for volatile
20 avoidance, 25 animals and 25 trials.

Eat-4 mutations reduced $G\alpha_c$ -induced cytotoxicity but not cell swelling

The *eat-4* gene was initially identified in screens for mutations that disrupted eating behavior (Avery, Genetics 133: 897-917, 1993). The *eat-4*
25 eating defect was caused by elimination of a glutamate-induced inhibitory synaptic signal (mediated by the M3 motor neuron), which could be observed in extracellular recordings of pharyngeal muscle activity (Raizen et al., Neuron

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12: 483-495, 1994). Given the results described herein, the eating defects and the ASH sensory defects could both be explained by an underlying defect in glutamate signaling.

To investigate this possibility, neurodegenerative phenotypes were examined as described above. In these experiments, *eat-4* mutations were found to be neuroprotective. The mutations significantly reduced $G\alpha_s$ -induced cytotoxicity but had no apparent effect on cell swelling, as indicated in Fig. 2. In addition to reducing cytotoxicity, the *eat-4* mutations also dramatically improved the locomotion rate of $\alpha_s(gf)$ animals. These results suggested that $G\alpha_s$ neurotoxicity was at least partially mediated by endogenous glutamate signaling.

Apoptosis was not required for $G\alpha_s$ neurotoxicity

Apoptosis is a naturally occurring process thought to play a critical role in the developing animal and is characterized morphologically by condensation of the chromatin followed by shrinkage of the cell body. Biochemically, DNA laddering, the degradation of nuclear DNA into oligonucleosomal fragments, is the hallmark of apoptosis. DNA laddering precedes cell death. Apoptosis is most likely dependent upon the activation of a cell death pathway. The best defined genetic pathway of cell death is in *C. elegans* where both effector (*ced-3* and *ced-4*) and repressor (*ced-9*) genes have been isolated. Similar genes have been identified in mammals. Whether excitotoxic death occurs by apoptosis or by necrosis has remained controversial. This uncertainty is primarily due to lack of genetic control of the apoptosis pathway in the previously described models for excitotoxicity.

In our experiments, we found that a mutation in the *ced-3* gene, which encodes an ICE protease and is required for apoptosis (Ellis and

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Horovitz, Cell 44: 817-829, 1986; Yuan et al., Cell 75: 641-652, 1993), had no effect on $G\alpha_s$ -induced swelling or killing (see Fig. 2). Thus, apoptosis was not required for $G\alpha_s$ -induced killing. However, in $\alpha_s(gf);unc-18$ double mutants, a significant fraction of PVC neurons had a highly condensed morphology, and these PVC neuron corpses appeared to be engulfed by surrounding hypodermal cells, both of which are characteristic of apoptotic deaths (Ellis et al., Ann. Rev. Cell Biol. 7: 663-698, 1991). As shown in Figs. 9A and 9B, in *unc-18* L1 larvae, 13% of the PVC neurons exhibit the condensed morphologies characteristic of programmed cell deaths, which was apparent in both bright field (Fig. 9A) and fluorescence (Fig. 9B) optics. In *unc-18* adults, 25% of the PVC neurons exhibited condensed morphologies and appeared to have been engulfed by surrounding hypodermal cells in the tail, as shown in Fig. 10A (bright field optics) and Fig. 10B (fluorescence optics). (Note that the position of the indicated cell body in Fig. 10B is much further posterior than in Fig. 7B).

$G\alpha_s$ neurotoxicity was concluded to be, in part, mediated by synaptic input to the dying cells, since *unc-18* mutations impair synaptic vesicle exocytosis (Gengyo-Ando, et al., Neuron 11: 703-711, 1993; Hata et al., Nature 366: 347-351, 1993). Furthermore, these results suggest that $G\alpha_s$ neurotoxicity occurs via two independent mechanisms. Synaptic input promotes an excitotoxic pattern of cell deaths; however, when synaptic input is impaired an apoptotic pattern emerges.

Screens for compounds that inhibit cAMP-based neurodegeneration

As described herein, constitutive activation of the GTP-binding protein $G\alpha_s$ induces a neurodegeneration phenotype that shares several properties with excitotoxic neuron loss in mammals. First, neuron loss occurs in two phases, whereby affected cells undergo a swelling response in young

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larvae, and subsequently die sometime during larval development. Second, neurons differ greatly in their susceptibility to $G\alpha_s$ -induced toxicity, ranging from 0-88% of cells affected. Third, a mutation that impairs the function of voltage-dependent calcium channels and one that reduces glutamate neurotransmission are neuroprotective.

The *acy-1* gene was identified in a screen for mutations that blocked G_s -induced killing and has been positionally cloned. The predicted ACY-1 protein (SEQ ID NO: 2) is highly similar (40% identical) to a mammalian adenylyl cyclase. Most consistent with this result is that $G\alpha_s$ -induced neurotoxicity is mediated by changes in cyclic adenosine monophosphate (cAMP) levels. Mutations that prevent programmed cell death, also known as apoptosis, do not prevent $G\alpha_s$ -induced neurotoxicity; however, when synaptic transmission was impaired (by an *unc-18* mutation), a subset of the deaths appear to become apoptotic. These experiments suggested that excitotoxicity normally occurs by both apoptosis and a second cytotoxic pathway. Given these results, screens for compounds that inhibit cAMP signaling may be carried out to identify drugs that alter cAMP-based neurodegeneration and to provide therapies to ameliorate these disorders in humans and other mammals. These assays may be carried out *in vivo* or *in vitro*, and a number of exemplary assays now follow.

a) *C. elegans* assays

The microscopic nematode, *C. elegans*, is a useful model for studying neurodegeneration because it allows researchers to observe changes in neuronal cells within the living organisms over the three days required for a *C. elegans* to develop from a single cell zygote to a mature adult. The biology of the *C. elegans* nervous system, which includes 302 neurons, has been well

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documented. Furthermore, there are several similarities between the *C. elegans* and human nervous systems. For example, many of the *C. elegans* neurotransmitters are the same as human neurotransmitters. In addition, many *C. elegans* genes used both inside and outside of the nervous system have counterparts in mammals.

To identify a compound capable of inhibiting cAMP-based neurotoxicity, candidate compounds are screened for an ability to alter cAMP levels using a *C. elegans* strain carrying a reporter transgene operably linked to a promoter of a gene that is either (i) regulated by cAMP or (ii) involved in cAMP regulation. Exemplary promoters include the *acy-1*, *unc-36*, and *eat-4* promoters. Other desirable promoters include any promoter from a nematode glutamate receptor (GluR) gene; such genes are listed, for example, in Table 3.

Table 3***C. elegans* glutamate signalling genes**

Gene Product	Genetic locus
iGluRs: C06E1.4 CO6A8.8 BO280.12 F41B4.4 C43H6 K10D3.1 ZC196.c	<i>glr-1</i>
GluCLs: GluCL α 1 GluCL β 1 ZC317.3 T10G3	
mGluRs: ZC506.4 F45H11	<i>mgr-1</i>

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Once constructed, a transgenic *C. elegans* strain carrying such a reporter gene is treated with a candidate compound, or any number of compounds in combination, and animals are screened for alterations in cAMP levels as reflected by alterations in the levels of reporter gene expression.

5 Useful reporter genes are those whose expression is detectable, preferably, using simple and rapid techniques. Preferable reporter genes include, without limitation, green fluorescent protein (*gfp*), spectrally shifted green fluorescent proteins (Rizzuto et al., Curr. Biol. 6: 183-188, 1996; Heim and Tsien, Curr. Biol. 6:178-182, 1996); *lacZ*, *her-1* (Perry et al., Gen. and
10 Dev. 7(2): 216-228, 1993), and *mec-4* (dominant) (Maricq et al., *supra*). Expression levels of these reporter genes may be directly measured by a variety of techniques known in the art. For example, if the reporter protein is a toxin (e.g., MEC-4), the expression level may be detected by measuring or observing cell viability. The expression level of a reporter protein with enzymatic activity
15 (e.g., *lacZ*) may be quantitated using colorimetric substrates (e.g., 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal)). And reporter gene products such as GFP may be screened directly by visual inspection.

If desired, reporter proteins may be fusion proteins that incorporate portions of the sequences involved in cAMP regulation, for example, the ACY-
20 1, UNC-36, or EAT-4 sequences. These fusion proteins are generated using nucleotide sequences and methods known in the art and described herein.

In one particular embodiment, such compound screens are carried out using rapid, high through-put assays. For example, transgenic *C. elegans* animals carrying *acy-1::gfp* reporter constructs are utilized. The animals are
25 distributed into 96-well microtiter dishes such that there is one animal per well. Candidate compounds are then individually or combinatorially added to the wells and assessed for an ability to reduce GFP expression as a means to test

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for an ability to inhibit cAMP-based neurodegeneration. GFP assays may be carried out by any means, but are preferably monitored using a microtiter plate fluorescence reader.

In an alternative compound screen, the reporter protein need not be GFP. For example, the transgenic animal may carry a *lacZ* reporter gene and be distributed into microtiter wells as described above. Following compound administration, transgenic animals are subjected to standard β -galactosidase activity assays described in the art (see, for example, Ausubel et al., *supra*). The Promega β -gactosidase enzyme assay system with reporter gene lysis buffer kit (Catalog # E2000) may be employed in this rapid high throughput 96 well assay system. By this method, reporter lysis buffer is added to each well. The *C. elegans* extracts are then incubated with the buffer and the o-nitrophenyl- β -D-glactopyranoside (OPTG) substrate provided in the kit. Optical density of the plate is then measured on a microtiter plate reader. Again, a reduced level of *lacZ* activity in a compound-treated well as compared to an untreated well indicates that the compound has an ability to inhibit cAMP-based neurodegeneration.

In addition, a variety of methods may be used in combination to screen for compounds capable of inhibiting cAMP-based neurodegeneration. For example, a *C. elegans* carrying two different expression constructs (e.g., the *acy-1* promoter operably linked to *gfp* and the *glr-1* promoter operably linked to *lacZ*) may be used to screen for a compound capable of inhibiting cAMP-based neurodegeneration by assaying for a reduction in the expression of both the *acy-1* and *eat-4* genes. In this assay, preferred compounds are capable of reducing the expression levels of both GFP and *lacZ*. However, a decrease in expression of one reporter gene (e.g., *gfp*), but not the other reporter gene (e.g., *lacZ*) identifies compounds capable of targeting particular

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components in a neurodegenerative pathway (in this case, the *acy-1* gene). Such compounds may be useful for treating particular types of neurodegenerative disorders.

In addition, nematode screens for compounds capable of inhibiting cAMP-based neurodegeneration may be based upon both neuroprotection and reporter gene expression. By this approach, for example, a transgenic *glr-1:: α_s (gf)* *C. elegans* is transformed with a second worm marker (e.g., the *acy-1::gfp* expression vector). Compound-treated *glr-1:: α_s (gf); acy-1::gfp* double transgenic animals are then screened, for example, for improved locomotion (i.e., a compound affecting the *glr-1* gene), reduction of GFP expression (i.e., a compound affecting the *acy-1* gene), or both (i.e., a compound affecting both the *glr-1* and *acy-1* genes) as compared to untreated *glr-1:: α_s (gf); acy-1::gfp* double transgenic animals. Again, gene-specific compounds may be useful for treating neurodegenerative disorders involving specific genes.

In yet another approach, compounds which affect neurodegenerative signals generated by a mammalian glutamate receptor may also be employed in a *C. elegans* screen. A large number of mammalian glutamate receptors (GluRs) have been previously described, and a comprehensive list of these proteins may be found in Hollmann and Heinemann (Ann. Rev. Neurosci. 17: 31-108, 1994). To carry out such a screen, the coding regions of one or more of these genes are inserted into a *C. elegans* expression vector such that the expression of the gene product is directed by the *glr-1* gene promoter. This construct is microinjected into a *glr:: α_s (gf)* transgenic *C. elegans*. Since only a subset of the *glr-1* expressing neurons die in such animals, any additional cell death (as measured, for example, by increased paralysis, neuronal swelling, or neurodegeneration) may be attributed to mammalian GluR expression. Candidate compounds are then administered to these animals, and differences

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observed in compound-treated animals versus untreated animals are used to identify a compound having an ability to affect mammalian GluR signalling. Again, compounds identified by this assay are useful for treating neurodegenerative disorders in a mammal.

5

b) Mammalian Cell Assays

Mammalian cells carrying a reporter gene operably linked to the promoter of a gene either regulated by cAMP or involved in cAMP regulation, for example, the mammalian homologues of the *acy-1*, *unc-36*, or *eat-4* genes, may also be used to screen for compounds that inhibit cAMP-based neurodegeneration. In one particular example, the promoter of the murine adenylyl cyclase type 9 encoding gene may be used to direct the expression of a reporter (e.g., GFP) in a mammalian expression vector. This vector is transfected into a mammalian cell by any of a number of different transfection methods well known in the art (e.g., electroporation, CaPO₄ precipitation, or DEAE-Dextran). Preferably, the mammalian cell is a mouse neuronal cell line, for example, a PC12 cell line. Candidate compounds are added to the culture medium of the transfected cells, and the level of expression of the reporter gene is measured and compared to a control, untreated cell line. A reduced level of reporter gene expression in a compound-treated cell line indicates that the compound has an ability to inhibit cAMP-based neurodegeneration in mammalian cells.

In addition, such a mammalian cell line may be transfected with more than one reporter gene operably linked to more than one cAMP regulatory gene promoter. For example, a mammalian cell transfected with a *gfp*-adenylyl cyclase type 9 construct may be doubly transfected with a construct comprising a mammalian *unc-36* promoter operably linked to a second reporter (e.g.,

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luciferase). Following addition of candidate compounds to the culture medium of doubly transfected cells, GFP expression is analyzed, for example, by flow cytometric analysis of half of the compound-treated cell population, and the remaining half is assayed for luciferase activity using known methods (e.g., the
5 luciferase assay kit commercially available from Promega). By comparing GFP and luciferase expression levels to those in untreated cells, a compound capable of altering cAMP-based neurodegeneration is identified. Furthermore, a compound capable of affecting, for example, the murine adenylyl cyclase type 9 gene but not the mammalian *unc-36* gene may also be isolated. Such a
10 compound may be useful for treating specific types of neurodegenerative disorders in mammals.

Alternatively, mammalian cells which endogenously express homologues of *C. elegans* genes involved in cAMP regulation or regulated by cAMP may be used to identify compounds capable of altering cAMP-induced
15 neurodegeneration. According to this method, following administration of a candidate compound, endogenous gene expression is measured by any of a variety of nucleic acid or immunological based assays including, without limitation, Northern blot, Western blot, and ELISA analyses. Compounds affecting endogenous gene expression levels as compared to untreated cells are
20 useful for treating cAMP-based neurodegeneration.

c) Animal Models

A number of animal models exist for the study of neurodegenerative disorders and find use in the screening methods described herein. For example,
25 such models may serve as a system in which to screen candidate compounds being tested *de novo* for an ability to alter cAMP-based neurodegeneration or as a secondary screen for testing compounds isolated in a *C. elegans*, yeast, or

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mammalian cell culture assay (for example, those assays described herein). Candidate compounds may be administered to animals prior to neurological damage to assay for an ability to prevent cAMP-based neurodegeneration. Alternatively, candidate compounds may be assessed for an ability to treat cAMP-based neurodegeneration following neurological insult. Animal models may also serve to determine the dosage requirement for an effective compound.

Particularly useful animal models include, without limitation, Parkinson's disease (PD) rat models, which are established by injecting the catecholamine-specific neurotoxin, 6-hydroxydopamine (6-OHDA), into the medial forebrain bundle or the substantia nigra pars compact to achieve a rapid degeneration of the nigrostriatal pathway, or into the striatum to achieve progressive degeneration, as has been described (see, for example Gerlach and Riederer, J. Neural. Transm. 103 (8-9): 987-1041, 1996; Bernard et al., J. Comp. Neurol. 368 (4): 553-568, 1996; Asada et al., Ex. Neurol. 139 (2): 173-187, 1996). Alternatively, rats may be rendered "epileptic" (i.e., induced to suffer brain seizures which often result in neuronal cell death) by administration of a variety of compounds including, for example, intravenous injection of bicuculline (Blennow et al., J. Cereb. Blood Flow Metab. 5: 439-445, 1995) or daily application of low intensity electrical stimulation. Finally, neuronal cell death which often results from stroke-induced ischemia may be mimicked by the 4-vessel occlusion experimental model described by Pulsinelli et al. (Ann. Neurol. 11: 491-498, 1982) and Francis and Pulsinelli (Brain Res. 243: 271-278, 1982).

d) Candidate inhibitors of cAMP-based neurodegeneration

A number of compounds have been shown to affect cAMP levels,

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and these provide good candidates for inhibitors of neurodegeneration. Such compounds are commercially available (e.g., from Research Biochemicals International) and include, without limitation, agonists of receptors that couple to Gi and inhibit adenylyl cyclases. Alpha 2 adrenergic receptor agonists (including B-HT 920 diHCl and Xylazine HCl), opioid delta receptor agonists (including [D-Ala2, D-Leu5]-enkephalin and [D-Pen2,5]-enkephalin), and D2 dopamine receptor agonists (including bromocriptine methane sulfonate and Quinelorane 2HCl) all inhibit adenylyl cyclases and may be assessed in screens described herein for an ability to inhibit cAMP-based neurodegeneration.

Therapeutics for treating human neurodegenerative disorders

A number of human neurological disorders are characterized by a loss of neurons through a degenerative process. Compounds isolated as described above based on their effect on cAMP levels are useful in treating these disorders. In addition, drugs known to lower cAMP levels are also useful therapeutics for treating, preventing, or slowing neurodegeneration. In particular, disorders that may be treated using such compounds include, without limitation, Parkinson's disease, Huntington's disease, Amyotrophic lateral sclerosis (ALS), Alzheimer's disease, multiple sclerosis, epilepsy, and stroke.

Compounds that alter cAMP levels may be administered by any appropriate route. For example, administration may be parenteral, intravenous, intra-arterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, by suppositories, or by oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or

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aerosols.

Methods well known in the art for making formulations are found, for example, in Remington's Pharmaceutical Sciences, (18th edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA. Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

Dosage is determined by standard techniques and is dependent, for example, upon the weight of the mammal and the type or extent of disorder being treated.

20 Diagnostics for neurodegenerative disorders

To determine whether an individual either has or is likely to develop a neurodegenerative disorder, that individual is screened for mutations in genes which are either involved in regulation of cAMP, or are regulated by cAMP, for example, genes encoding the adenylyl cyclases, G proteins, or human homologues of UNC-36 or EAT-4 proteins described herein. Such assays may be carried out by any standard technique including, without limitation, methods involving sequencing or mismatch binding or cleaving assays. In one particular

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example, a nucleic acid sample derived from the neuronal cells of an individual may be isolated (for example, by PCR amplification), and a cAMP regulatory gene (or a portion thereof) subjected to rapid sequence analysis by automated sequencing techniques using primers generated from sequences described
5 herein and in the art.

Alternatively, an individual who either has or is likely to develop a neurodegenerative disorder may be screened for altered expression of adenylyl cyclases, G proteins, or the human homologues of UNC-36 or EAT-4 proteins, or for an increased level of cellular cAMP, particularly in neuronal cells. Such
10 assays may be carried out, for example, using any standard nucleic acid-based assay (e.g., Northern blot analysis) or immunological assay (e.g., enzyme-linked immunosorbent assay (ELISA)), preferably in a high through-put assay format. In one particular example, neuronal cells obtained from an individual
being screened for a neurodegenerative disorder may be isolated and analyzed
15 for the expression of adenylyl cyclases, G proteins, and the human homologues of UNC-36 and EAT-4 proteins by ELISAs using fluorophore-tagged antibodies directed toward these proteins as probes. Individuals incapable of expressing certain proteins may be identified by rapidly assessing the results of these ELISAs in a microtiter plate format.

20 In particular examples, candidate human genes, for example, those involved in cAMP regulation, are examined for genetic linkage to heritable forms of neurodegeneration found in humans or, as a model system, mice. These genetic linkages are assessed using standard methods known in the art, and, upon identification of a linkage with neurodegeneration, diagnostic
25 mutation detection is conducted as described herein. Listed in Table 4 are exemplary candidate human genes likely involved in neurodegeneration.

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Table 4**Candidate Human Neurodegeneration Genes**

Class of Protein	Gene Product
Phosphodiesterases	PDE4A PDE4B PDE6G PDE7A
5 G alpha subunits	GNAS1 GNAI1 GNAI2 GNAI3 Golf
Protein Phosphatases	PPP1CB PPP1CC PPP2CA PPP2R4 PPP2R5A PPP2R5C PPP2R5D PPP2R5E PPP3CA PPP3CB PPP3R1

Methods for isolating genes involved in neurodegeneration10 a) *C. elegans* Screens

Additional genes involved in neurodegeneration may be isolated using the methods described herein. For example, a gene involved in neurodegeneration may be isolated by inducing paralysis and neurodegeneration in *C. elegans*. This is accomplished, for example, by

15 generating a nematode strain carrying a constitutively active (GTP-ase

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defective) $G\alpha_s$ subunit gene operably linked to a glutamate receptor (GluR) promoter, such as *glr-1*. The transgenic *C. elegans* is screened for gene mutations which restore locomotion and reduce neurodegeneracy (cytotoxicity genes) or which reduce $G\alpha_s$ -induced neuronal cell swelling (swelling genes).

5 I. $G\alpha_s$ -associated Cytotoxicity Genes

To isolate a $G\alpha_s$ -associated cytotoxicity gene, *glr-1:: α_s (gf)* transgenic nematodes are mutagenized, for example, with EMS or γ -irradiation, and then screened for mutants with both improved locomotion and increased survival of the $G\alpha_s$ expressing neurons. If desired, these mutants may be genetically mapped and placed into complementation groups. The genes identified in these
10 mutants may then be positionally cloned.

 II. $G\alpha_s$ -associated Swelling Genes

To isolate a $G\alpha_s$ -associated swelling gene, *glr-1:: α_s (gf)* transgenic nematodes are mutagenized, for example, with EMS or γ -irradiation. First
15 stage larvae are then isolated and screened by fluorescence microscopy (as described herein) for mutants which show a reduced incidence of swelling of $G\alpha_s$ -expressing neurons. These mutants may be genetically mapped and positionally cloned.

 III. Other Genes

20 The transgenic animals developed to identify compounds that inhibit cAMP-based neurodegeneration may also be used to identify additional genes involved in neurodegeneration. For example, *C. elegans* doubly transgenic for *acy-1::gfp*; *glr-1:: α_s (gf)* may be mutagenized (for example, with EMS or γ -irradiation) and then analyzed for restored locomotion and reduced
25 neurodegeneration (i.e., for a mutation in a gene which affects the *glr-1* promoter) or a reduced level of GFP expression (i.e., for a mutation in a gene which affects the *acy-1* promoter), or both (i.e., a mutation in a gene which

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affects both *acy-1* and *glr-1* gene promoters).

In an alternative approach, physiological stresses, such as ischemia, due to, for example, the interruption of available oxygen, may be administered to EMS or γ -irradiated worms to induce neurodegeneration. Mutants which
5 resist ischemia-induced neurodegeneration may then be isolated and characterized to identify the neuroprotective mutant gene.

A gene involved in neurodegeneration may be cloned and sequenced by standard methods (see, for example, Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1994 and Sambrook,
10 Fritsch and Maniatis, *Molecular Cloning: A Laboratory Manual (2d ed.)*, CSH Press, 1989). If desired, a protein product from this gene may then be produced, for example, by inserting the cloned gene into an expression vector and introducing this vector into bacterial or eukaryotic cells to produce recombinant proteins. Techniques for such manipulations are disclosed in
15 Sambrook et al., *supra*, and are well known in the art. Genes involved in neurodegeneration or their protein products may be used in any of the screening or diagnostic assays described herein.

b) Yeast Screens

20 Another approach to identify genes involved in neurodegeneration utilizes yeast carrying a reporter gene operably linked to a promoter from a cAMP regulatory gene, and, preferably, a mammalian cAMP regulatory gene. The reporter construct is stably introduced into yeast by any standard method. A cDNA library (preferably, from a mammalian cell) is then introduced into
25 the yeast carrying the reporter construct, and yeast colonies exhibiting an increased level of reporter gene expression (e.g., *lacZ* reporter yeast with increased blue colony color on X-Gal) are identified. Such yeast carry a cDNA

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capable of binding to the cAMP promoter and are therefore good candidates for a gene involved in cAMP-based neurodegeneration. If desired, the promoter sequences from the newly isolated gene may also be used to generate reporter cells (e.g., reporter yeast or transgenic *C. elegans*) to identify additional genes involved in cAMP-based neurodegeneration.

Moreover, this yeast system may be used to screen for compounds which inhibit the ability of the cDNA to induce reporter gene expression. Such compounds provide good candidates for therapeutics for treating cAMP-based neurodegeneration.

Mammalian genes involved in neurodegeneration

a) Mammalian *eat-4* genes

Any of a variety of procedures well known in the art may be utilized to clone the mammalian homologues of the nematode *eat-4* gene, and one so skilled will routinely adapt one of these methods in order to obtain the desired gene.

One such method for obtaining a mammalian gene sequence is to use an oligonucleotide probe generated by the *C. elegans eat-4* gene sequence to screen a mammalian cDNA or genomic DNA library for sequences which hybridize to the probe. Hybridization techniques are well known to the skilled artisan, and are described, for example, in Ausubel et al., *supra*, and Sambrook et al., *supra*. cDNA or genomic DNA library preparation is also well known in the art. A large number of prepared nucleic acid libraries are also commercially available. The oligonucleotide probes are readily designed using the sequences described herein and standard techniques. The oligonucleotide probes may be based upon the sequence of either strand of DNA encoding the *eat-4* gene product (SEQ ID NO: 1). Exemplary oligonucleotide probes are

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degenerate probes (i.e., a mixture of all possible coding sequences for the EAT-4 protein).

If desired, the cloned gene may be inserted into an expression vector and introduced into bacterial or eukaryotic cells to produce the mammalian EAT-4 protein. Techniques for such manipulations are disclosed, for example, in Sambrook et al., *supra*. The mammalian *eat-4* gene or gene product may be used in the neurodegeneration screening or diagnostic assays described herein.

b) *eat-4* related *C. elegans* genes

Genes related to *eat-4* may be isolated by methods similar to those described above. For example, a cosmid library from *C. elegans* may be screened with the degenerate oligonucleotide probes described above under low stringency hybridization conditions to isolate *eat-4* related *C. elegans* genes. Oligonucleotide probes may be prepared from these gene sequences and may be used to screen mammalian nucleic acid libraries for hybridizing sequences, thus, identifying mammalian homologues of these *eat-4* related genes.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention

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pertains and may be applied to the essential features hereinbefore set forth, and follows in the scope of the appended claims.

Other embodiments are within the claims.

What is claimed is:

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SEQUENCE LISTING

(1) GENERAL INFORMATION

- (i) APPLICANT: The General Hospital Corporation
- (ii) TITLE OF THE INVENTION: METHODS FOR THE DETECTION, TREATMENT, AND PREVENTION OF NEURODEGENERATION
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Clark & Elbing LLP
 - (B) STREET: 176 Federal Street
 - (C) CITY: Boston
 - (D) STATE: MA
 - (E) COUNTRY: USA
 - (F) ZIP: 02110
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette
 - (B) COMPUTER: IBM Compatible
 - (C) OPERATING SYSTEM: DOS
 - (D) SOFTWARE: FastSEQ for Windows Version 2.0
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE: 29-MAY-1998
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/864,785
 - (B) FILING DATE: 29-MAY-1997
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Elbing, Karen L
 - (B) REGISTRATION NUMBER: 35,238
 - (C) REFERENCE/DOCKET NUMBER: 00786/353WO1
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 617-428-0200
 - (B) TELEFAX: 617-428-7045
 - (C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 576 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met	Ser	Ser	Trp	Asn	Glu	Ala	Trp	Asp	Arg	Gly	Lys	Gln	Met	Val	Gly
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Glu	Pro	Leu	Ala	Lys	Met	Thr	Ala	Ala	Ala	Ala	Ser	Ala	Thr	Gly	Ala
			20					25					30		
Ala	Pro	Pro	Gln	Gln	Met	Gln	Glu	Gly	Asn	Glu	Asn	Pro	Met	Gln	
		35					40				45				
Met	His	Ser	Asn	Lys	Val	Leu	Gln	Val	Met	Glu	Gln	Thr	Trp	Ile	Gly
						55					60				
Lys	Cys	Arg	Lys	Arg	Trp	Leu	Leu	Ala	Ile	Leu	Ala	Asn	Met	Gly	Phe
65					70					75				80	
Met	Ile	Ser	Phe	Gly	Ile	Arg	Cys	Asn	Phe	Gly	Ala	Ala	Lys	Thr	His
				85					90					95	
Met	Tyr	Lys	Asn	Tyr	Thr	Asp	Pro	Tyr	Gly	Lys	Val	His	Met	His	Glu
			100					105					110		
Phe	Asn	Trp	Thr	Ile	Asp	Glu	Leu	Ser	Val	Met	Glu	Ser	Ser	Tyr	Phe
			115				120					125			
Tyr	Gly	Tyr	Leu	Val	Thr	Gln	Ile	Pro	Ala	Gly	Phe	Leu	Ala	Ala	Lys
			130			135					140				
Phe	Pro	Pro	Asn	Lys	Leu	Phe	Gly	Phe	Gly	Ile	Gly	Val	Gly	Ala	Phe
145					150					155				160	
Leu	Asn	Ile	Leu	Leu	Pro	Tyr	Gly	Phe	Lys	Val	Lys	Ser	Asp	Tyr	Leu
				165					170					175	
Val	Ala	Phe	Ile	Gln	Ile	Thr	Gln	Gly	Leu	Val	Gln	Gly	Val	Cys	Tyr
			180					185					190		
Pro	Ala	Met	His	Gly	Val	Trp	Arg	Tyr	Trp	Ala	Pro	Pro	Met	Glu	Arg
		195					200					205			
Ser	Lys	Leu	Ala	Thr	Thr	Ala	Phe	Thr	Gly	Ser	Tyr	Ala	Gly	Ala	Val
		210				215					220				
Leu	Gly	Leu	Pro	Leu	Ser	Ala	Phe	Leu	Val	Ser	Tyr	Val	Ser	Trp	Ala
225					230					235				240	
Ala	Pro	Phe	Tyr	Leu	Tyr	Gly	Val	Cys	Gly	Val	Ile	Trp	Ala	Ile	Leu
				245					250					255	
Trp	Phe	Cys	Val	Thr	Phe	Glu	Lys	Pro	Ala	Phe	His	Pro	Thr	Ile	Ser
			260					265					270		
Gln	Glu	Glu	Lys	Ile	Phe	Ile	Glu	Asp	Ala	Ile	Gly	His	Val	Ser	Asn
		275					280					285			
Thr	His	Pro	Thr	Ile	Arg	Ser	Ile	Pro	Trp	Lys	Ala	Ile	Val	Thr	Ser
		290				295					300				
Lys	Pro	Val	Trp	Ala	Ile	Ile	Val	Ala	Asn	Phe	Ala	Arg	Ser	Trp	Thr
305					310					315				320	
Phe	Tyr	Leu	Leu	Leu	Gln	Asn	Gln	Leu	Thr	Tyr	Met	Lys	Glu	Ala	Leu
				325					330					335	
Gly	Met	Lys	Ile	Ala	Asp	Ser	Gly	Leu	Leu	Ala	Ala	Ile	Pro	His	Leu
			340					345					350		
Val	Met	Gly	Cys	Val	Val	Leu	Met	Gly	Gly	Gln	Leu	Ala	Asp	Tyr	Leu
		355					360					365			
Arg	Ser	Asn	Lys	Ile	Leu	Ser	Thr	Thr	Ala	Val	Arg	Lys	Ile	Phe	Asn
		370				375					380				
Cys	Gly	Gly	Phe	Gly	Gly	Glu	Ala	Ala	Phe	Met	Leu	Ile	Val	Ala	Tyr
385					390					395				400	
Thr	Thr	Ser	Asp	Thr	Thr	Ala	Ile	Met	Ala	Leu	Ile	Ala	Ala	Val	Gly
				405					410					415	
Met	Ser	Gly	Phe	Ala	Ile	Ser	Gly	Phe	Asn	Val	Asn	His	Leu	Asp	Ile
			420					425					430		
Ala	Pro	Arg	Tyr	Ala	Ala	Ile	Leu	Met	Gly	Phe	Ser	Asn	Gly	Ile	Gly
		435					440						445		

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Thr Leu Ala Gly Leu Thr Cys Pro Phe Val Thr Glu Ala Phe Thr Ala
 450                      455                      460
His Ser Lys His Gly Trp Thr Ser Val Phe Leu Leu Ala Ser Leu Ile
465                      470                      475                      480
His Phe Thr Gly Val Thr Phe Tyr Ala Val Tyr Ala Ser Gly Glu Leu
                      485                      490                      495
Gln Glu Trp Ala Glu Pro Lys Glu Glu Glu Trp Ser Asn Lys Glu
          500                      505                      510
Leu Val Asn Lys Thr Gly Ile Asn Gly Thr Gly Tyr Gly Ala Ala Glu
          515                      520                      525
Thr Thr Phe Thr Gln Leu Pro Ala Gly Val Asp Ser Ser Tyr Gln Ala
          530                      535                      540
Gln Ala Ala Pro Ala Pro Gly Thr Asn Pro Phe Ala Ser Ala Trp Asp
545                      550                      555                      560
Glu His Gly Ser Ser Gly Val Val Glu Asn Pro His Tyr Gln Gln Trp
          565                      570                      575

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1253 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Asp Asp Asp Val Gly Glu Arg Thr Pro Ala Leu Gly Gly Ser Cys
 1          5          10          15
Gly Pro Ser Val Arg Ala His Ser Ser Pro Arg Arg Val Pro Leu
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Phe Glu Arg Ala Ser Ala Arg Trp Trp Asn Pro Gln Phe Arg Ser Ala
          35          40          45
Thr Leu Glu Ala Gln Tyr Trp Lys Cys Ser Phe Ser Gln Leu Arg Asp
          50          55          60
Arg Phe Arg Ser Gly Leu Ile Tyr Ile Ala Val Val Ile Ala Ala Trp
65          70          75          80
Thr Leu Tyr Leu Ala Leu Phe Asp Arg Thr Phe Ile Gln His Trp Ile
          85          90          95
Val Ser Leu Cys Leu Cys Ala Ile Ile Phe Ala Met Phe Ala Phe Thr
          100          105          110
Ala Cys Ala Ala Gln Tyr Gln Arg Phe Tyr Met Pro Thr Ser Phe Leu
          115          120          125
Cys Thr Phe Leu Ile Cys Leu Val Thr Leu Leu Ile Phe Ser Ala Glu
          130          135          140
Asn Gln Ala Ala Phe Met Thr Pro Val Ala Ser Leu Ala Thr Ser Phe
145          150          155          160
Gln Val Val Leu Leu Ile Tyr Thr Val Ile Pro Leu Pro Leu Tyr Leu
          165          170          175
Cys Ile Leu Ile Gly Ile Ile Tyr Ser Ile Leu Phe Glu Ile Leu Asn
          180          185          190
Lys Asn Lys Ile Gly Leu Glu Glu Ala Gly Tyr Ile Lys Leu Val Leu
          195          200          205
His Ala Gly Val His Leu Leu Gly Val His Leu Phe Ile Leu Thr Gln
          210          215          220
Val Arg Gln Arg Lys Thr Phe Leu Lys Val Gly Gln Ser Met Leu Ala

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Ser	Val	Met	Pro	Lys	Lys	Val	Ala	Asp	Glu	Leu	Leu	Lys	Asp	Ala	Ser
			260					265					270		
Glu	Leu	Arg	Arg	Pro	Ser	Ala	Ser	Asn	Asp	Ser	Asn	Cys	Arg	Thr	Ser
		275					280					285			
Asn	Ala	Thr	Gln	Val	Asp	Gln	Pro	Leu	Ala	Lys	Met	Val	Pro	Glu	Tyr
	290					295					300				
Arg	Lys	Phe	Arg	Pro	Phe	Thr	Met	Asn	Leu	Met	Thr	Asn	Val	Ser	Ile
305					310					315				320	
Leu	Phe	Ala	Asp	Ile	Ala	Gly	Phe	Thr	Lys	Met	Ser	Ser	Asn	Lys	Ser
			325						330					335	
Ala	Asp	Glu	Leu	Val	Asn	Leu	Leu	Asn	Asp	Leu	Phe	Gly	Arg	Phe	Asp
			340					345					350		
Thr	Leu	Cys	Arg	Leu	Arg	Gly	Leu	Glu	Lys	Ile	Ser	Thr	Leu	Gly	Asp
		355					360					365			
Cys	Tyr	Tyr	Cys	Val	Ala	Gly	Cys	Pro	Glu	Pro	Cys	Asp	Asp	His	Ala
	370					375					380				
Cys	Arg	Thr	Val	Glu	Met	Gly	Leu	Asp	Met	Ile	Val	Ala	Ile	Arg	Gln
385					390					395				400	
Phe	Asp	Ile	Asp	Arg	Gly	Gln	Glu	Val	Asn	Met	Arg	Val	Gly	Ile	His
			405					410					415		
Thr	Gly	Lys	Val	Met	Cys	Gly	Met	Val	Gly	Thr	Lys	Arg	Phe	Lys	Phe
			420					425					430		
Asp	Val	Phe	Ser	Asn	Asp	Val	Thr	Leu	Ala	Asn	Glu	Met	Glu	Ser	Ser
	435					440					445				
Gly	Val	Ala	Gly	Arg	Val	His	Val	Ser	Glu	Ala	Thr	Ala	Lys	Leu	Leu
	450					455					460				
Lys	Gly	Leu	Tyr	Glu	Ile	Glu	Glu	Gly	Pro	Asp	Tyr	Asp	Gly	Pro	Leu
465					470				475					480	
Arg	Met	Gln	Val	Gln	Gly	Thr	Glu	Arg	Arg	Val	Lys	Pro	Glu	Ser	Met
			485					490					495		
Lys	Thr	Phe	Phe	Ile	Lys	Gly	Arg	Ile	Asn	Asp	Gly	Val	Glu	Glu	Glu
		500						505					510		
Val	Met	Gln	Val	Gln	Glu	Val	Glu	Ser	Leu	His	Ser	Gln	Lys	Ser	Ser
	515						520					525			
Lys	Lys	Ser	Thr	Leu	Lys	Gln	Lys	Trp	Ala	Glu	Lys	Leu	Lys	Met	Asn
	530					535					540				
His	Thr	Asn	Ser	Tyr	Pro	Met	Arg	Ala	Ala	Ala	Arg	Glu	Gly	Gly	Gly
545					550				555					560	
Ser	Leu	Arg	Ile	Lys	Leu	Ala	Glu	Arg	Asn	Arg	Ser	Thr	Gln	Leu	Leu
			565					570					575		
Pro	Lys	Glu	Ser	Asn	Ser	Ile	Cys	Ile	Met	Glu	Asp	Asn	Arg	Lys	Ser
		580						585					590		
Ala	Ser	Leu	Gln	Ala	Leu	Ala	Thr	Asn	Asn	Phe	Asn	Gly	Ser	Asn	Thr
	595						600					605			
Asp	Thr	Asn	Asn	Thr	Tyr	Ser	Glu	Arg	Gly	Val	Ala	Gly	Ser	Val	Ser
	610					615					620				
Lys	Lys	Ser	Val	Ala	Gly	Ser	Glu	Ser	Asn	Ser	Ile	Lys	Gly	Ser	Arg
625					630				635					640	
Ser	Ser	Gly	Leu	Gln	Leu	Ser	Leu	Gln	Asp	Gly	Asn	Ser	Asp	Leu	Asn
			645					650					655		
Ser	Val	Gly	Gly	Leu	Asp	Thr	Ala	Ile	Ser	His	His	His	Asn	Ala	Ala

BNSDOCID: <WO 9853856A1_IA>

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          1125          1130          1135
Gln His Val Leu Ser Val Phe Asn Glu Asp Leu Leu Asn Phe Asp Phe
          1140          1145          1150
Val Cys Lys Leu Gly Leu Asn Ile Gly Pro Val Thr Ala Gly Val Ile
          1155          1160          1165
Gly Thr Thr Lys Leu Tyr Tyr Asp Ile Trp Gly Asp Thr Val Asn Ile
          1170          1175          1180
Ala Ser Arg Met Tyr Ser Thr Gly Val Leu Asn Arg Ile Gln Val Ser
185          1190          1195          1200
Gln His Thr Arg Glu Tyr Leu Leu Asp Arg Tyr Glu Phe Glu Phe Arg
          1205          1210          1215
Asp His Ile Glu Val Lys Gly Ile Asp Gly Gly Met Asp Thr Tyr Leu
          1220          1225          1230
Leu Val Gly Arg Lys Gly Asp Gly Ile Pro Pro Ser Ile Lys Asp Asn
          1235          1240          1245
Gln Glu Asp Glu Phe
          1250

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1400 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

Met Ala Ser Ser Pro His Gln Gln Leu Leu His His His Ser Thr Glu
 1          5          10          15
Val Ser Cys Asp Ser Ser Gly Asp Ser Asn Ser Val Arg Val Lys Ile
          20          25          30
Asn Pro Lys Gln Leu Ser Ser Asn Thr His Pro Lys His Cys Lys Tyr
          35          40          45
Ser Ile Ser Ser Ser Cys Ser Ser Ser Gly Asp Ser Gly Gly Leu Pro
          50          55          60
Arg Arg Val Gly Gly Gly Gly Arg Leu Arg Arg Gln Lys Lys Leu Pro
65          70          75          80
Gln Leu Phe Glu Arg Ala Ser Ser Arg Trp Trp Asp Pro Lys Phe Asp
          85          90          95
Ser Met Asn Leu Glu Glu Ala Cys Leu Glu Arg Cys Phe Pro Gln Thr
          100          105          110
Gln Arg Arg Phe Arg Tyr Ala Leu Phe Tyr Val Gly Phe Ala Cys Leu
          115          120          125
Leu Trp Ser Ile Tyr Phe Ala Val His Met Lys Ser Lys Val Ile Val
          130          135          140
Met Val Val Pro Ala Leu Cys Phe Leu Val Val Cys Val Gly Phe Phe
145          150          155          160
Leu Phe Thr Phe Thr Lys Leu Tyr Ala Arg His Tyr Ala Trp Thr Ser
          165          170          175
Leu Ala Leu Thr Leu Leu Val Phe Ala Leu Thr Leu Ala Ala Gln Phe
          180          185          190
Gln Val Trp Thr Pro Leu Ser Gly Arg Val Asp Ser Ser Asn His Thr
          195          200          205
Leu Thr Ala Thr Pro Ala Asp Thr Cys Leu Ser Gln Val Gly Ser Phe
          210          215          220

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Ser Ile Cys Ile Glu Val Leu Leu Leu Leu Tyr Thr Val Met Gln Leu
 225 230 235 240
 Pro Leu Tyr Leu Ser Leu Phe Leu Gly Val Val Tyr Ser Val Leu Phe
 245 250 255
 Glu Thr Phe Gly Tyr His Phe Arg Asn Glu Asp Cys Tyr Pro Ser Pro
 260 265 270
 Gly Pro Gly Ala Leu His Trp Glu Leu Leu Ser Arg Ala Leu Leu His
 275 280 285
 Val Cys Ile His Ala Ile Gly Ile His Leu Phe Val Met Ser Gln Val
 290 295 300
 Arg Ser Arg Ser Thr Phe Leu Lys Val Gly Gln Ser Ile Met His Gly
 305 310 315 320
 Lys Asp Leu Glu Val Glu Lys Ala Leu Lys Glu Arg Met Ile His Ser
 325 330 335
 Val Met Pro Arg Ile Ile Ala Asp Asp Leu Met Lys Gln Gly Asp Glu
 340 345 350
 Glu Ser Glu Asn Ser Val Lys Arg His Ala Thr Ser Ser Pro Lys Asn
 355 360 365
 Arg Lys Lys Lys Ser Ser Ile Gln Lys Ala Pro Ile Ala Phe Arg Pro
 370 375 380
 Phe Lys Met Gln Gln Ile Glu Glu Val Ser Ile Leu Phe Ala Asp Ile
 385 390 395 400
 Val Gly Phe Thr Lys Met Ser Ala Asn Lys Ser Ala His Ala Leu Val
 405 410 415
 Gly Leu Leu Asn Asp Leu Phe Gly Arg Phe Asp Arg Leu Cys Glu Gln
 420 425 430
 Thr Lys Cys Glu Lys Ile Ser Thr Leu Gly Asp Cys Tyr Tyr Cys Val
 435 440 445
 Ala Gly Cys Pro Glu Pro Arg Ala Asp His Ala Tyr Cys Cys Ile Glu
 450 455 460
 Met Gly Leu Gly Met Ile Lys Ala Ile Glu Gln Phe Cys Gln Glu Lys
 465 470 475 480
 Lys Glu Met Val Asn Met Arg Val Gly Val His Thr Gly Thr Val Leu
 485 490 495
 Cys Gly Ile Leu Gly Met Arg Arg Phe Lys Phe Asp Val Trp Ser Asn
 500 505 510
 Asp Val Asn Leu Ala Asn Leu Met Glu Gln Leu Gly Val Ala Gly Lys
 515 520 525
 Val His Ile Ser Glu Ala Thr Ala Lys Tyr Leu Asp Asp Arg Tyr Glu
 530 535 540
 Met Glu Asp Gly Arg Val Ile Glu Arg Leu Gly Gln Ser Val Val Ala
 545 550 555 560
 Asp Gln Leu Lys Gly Leu Lys Thr Tyr Leu Ile Ser Gly Gln Arg Ala
 565 570 575
 Lys Glu Ser His Cys Ser Cys Ala Glu Ala Leu Leu Ser Gly Phe Glu
 580 585 590
 Val Ile Asp Asp Ser Arg Glu Ser Ser Gly Pro Arg Gly Gln Gly Thr
 595 600 605
 Ala Ser Pro Gly Ser Val Ser Asp Leu Ala Gln Thr Val Lys Thr Phe
 610 615 620
 Asp Asn Leu Lys Thr Cys Pro Ser Cys Gly Ile Thr Phe Ala Pro Lys
 625 630 635 640
 Ser Glu Ala Gly Ala Glu Gly Gly Thr Val Gln Asn Gly Cys Gln Asp
 645 650 655
 Glu Pro Lys Thr Ser Thr Lys Ala Ser Gly Gly Pro Asn Ser Lys Thr
 660 665 670
 Gln Asn Gly Leu Leu Ser Pro Pro Ala Glu Glu Lys Leu Thr Asn Ser
 675 680 685

-45-

Gln Thr Ser Leu Cys Glu Ile Leu Gln Glu Lys Gly Arg Trp Ala Gly
 690 695 700
 Val Ser Leu Asp Gln Ser Ala Leu Leu Pro Leu Arg Phe Lys Asn Ile
 705 710 715 720
 Arg Glu Lys Thr Asp Ala His Phe Val Asp Val Ile Lys Glu Asp Ser
 725 730 735
 Leu Met Lys Asp Tyr Phe Phe Lys Pro Pro Ile Asn Gln Phe Ser Leu
 740 745 750
 Asn Phe Leu Asp Gln Glu Leu Glu Arg Ser Tyr Arg Thr Ser Tyr Gln
 755 760 765
 Glu Glu Val Ile Lys Asn Ser Pro Val Lys Thr Phe Ala Ser Ala Thr
 770 775 780
 Phe Ser Ser Leu Leu Asp Val Phe Leu Ser Thr Thr Val Phe Leu Ile
 785 790 795 800
 Leu Ser Ile Thr Cys Phe Leu Lys Tyr Gly Ala Thr Ala Thr Pro Pro
 805 810 815
 Pro Pro Ala Ala Leu Ala Val Phe Gly Ala Asp Leu Leu Leu Glu Val
 820 825 830
 Leu Ser Leu Ile Val Ser Ile Arg Met Val Phe Phe Leu Glu Asp Val
 835 840 845
 Met Thr Cys Thr Lys Trp Leu Leu Glu Trp Ile Ala Gly Trp Leu Pro
 850 855 860
 Arg His Cys Ile Gly Ala Ile Leu Val Ser Leu Pro Ala Leu Ala Val
 865 870 875 880
 Tyr Ser His Ile Thr Ser Glu Phe Glu Thr Asn Ile His Val Thr Met
 885 890 895
 Phe Thr Gly Ser Ala Val Leu Val Ala Val Val His Tyr Cys Asn Phe
 900 905 910
 Cys Gln Leu Ser Ser Trp Met Arg Ser Ser Leu Ala Thr Ile Val Gly
 915 920 925
 Ala Gly Leu Leu Leu Leu Leu His Ile Ser Leu Cys Gln Asp Ser Ser
 930 935 940
 Ile Val Met Ser Pro Leu Asp Ser Ala Gln Asn Phe Ser Ala Gln Arg
 945 950 955 960
 Asn Pro Cys Asn Ser Ser Val Leu Gln Asp Gly Arg Arg Pro Ala Ser
 965 970 975
 Leu Ile Gly Lys Glu Leu Ile Leu Thr Phe Phe Leu Leu Leu Leu
 980 985 990
 Val Trp Phe Leu Asn Arg Glu Phe Glu Val Ser Tyr Arg Leu His Tyr
 995 1000 1005
 His Gly Asp Val Glu Ala Asp Leu His Arg Thr Lys Ile Gln Ser Met
 1010 1015 1020
 Arg Asp Gln Ala Asp Trp Leu Leu Arg Asn Ile Ile Pro Tyr His Val
 1025 1030 1035 1040
 Ala Glu Gln Leu Lys Val Ser Gln Thr Tyr Ser Lys Asn His Asp Ser
 1045 1050 1055
 Gly Gly Val Ile Phe Ala Ser Ile Val Asn Phe Ser Glu Phe Tyr Glu
 1060 1065 1070
 Glu Asn Tyr Glu Gly Gly Lys Glu Cys Tyr Arg Val Leu Asn Glu Leu
 1075 1080 1085
 Ile Gly Asp Phe Asp Glu Leu Leu Ser Lys Pro Asp Tyr Asn Ser Ile
 1090 1095 1100
 Glu Lys Ile Lys Thr Ile Gly Ala Thr Tyr Met Ala Ala Ser Gly Leu
 1105 1110 1115 1120
 Asn Thr Ala Gln Cys Gln Glu Gly Gly His Pro Gln Glu His Leu Arg
 1125 1130 1135
 Ile Leu Phe Glu Phe Ala Lys Glu Met Met Arg Val Val Asp Asp Phe
 1140 1145 1150

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Asn Asn Asn Met Leu Trp Phe Asn Phe Lys Leu Arg Val Gly Phe Asn
    1155                1160                1165
His Gly Pro Leu Thr Ala Gly Val Ile Gly Thr Thr Lys Leu Leu Tyr
    1170                1175                1180
Asp Ile Trp Gly Asp Thr Val Asn Ile Ala Ser Arg Met Asp Thr Thr
185                1190                1195                1200
Gly Val Glu Cys Arg Ile Gln Val Ser Glu Glu Ser Tyr Arg Val Leu
                1205                1210                1215
Ser Lys Met Gly Tyr Asp Phe Asp Tyr Arg Gly Thr Val Asn Val Lys
                1220                1225                1230
Gly Lys Gly Gln Met Lys Thr Tyr Leu Tyr Pro Lys Cys Thr Asp Asn
                1235                1240                1245
Gly Val Val Pro Gln His Gln Leu Ser Ile Ser Pro Asp Ile Arg Val
1250                1255                1260
Gln Val Asp Gly Ser Ile Gly Arg Ser Pro Thr Asp Glu Ile Ala Asn
265                1270                1275                1280
Leu Val Pro Ser Val Gln Tyr Ser Asp Lys Ala Ser Leu Gly Ser Asp
                1285                1290                1295
Asp Ser Thr Gln Ala Lys Glu Ala Arg
    1300                1305

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Claims

1. A method for identifying a compound to treat or prevent the onset of a neurodegenerative disorder, said method comprising the steps of:
 - a) providing a cell comprising a reporter gene operably linked to a
5 cAMP regulatory gene or promoter;
 - b) contacting said cell with a candidate compound; and
 - c) measuring expression of said reporter gene, a change in said expression in response to said candidate compound identifying a compound that is useful to treat or prevent the onset of a neurodegenerative disorder.
- 10 2. The method of claim 1, wherein said cAMP regulatory gene is an *acy-1* gene, an *eat-4* gene, an *unc-36* gene, or a glutamate receptor-encoding gene.
- 15 3. The method of claim 1, wherein said change in said expression is a decrease in expression.
4. The method of claim 1, wherein said cell is present in an animal.
- 20 5. The method of claim 4, wherein said animal is *C. elegans* or a rodent.
6. The method of claim 1, wherein said neurodegenerative disorder is Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis,
25 multiple sclerosis, Alzheimer's disease, stroke, or epilepsy.
7. A cell for identifying a compound to treat or prevent the onset of

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a neurodegenerative disorder, said cell comprising a reporter gene operably linked to a promoter of a cAMP regulatory gene.

8. The cell of claim 7, wherein said cell is present in an animal.

5

9. The cell of claim 8, wherein said animal is *C. elegans* or a rodent.

10. The cell of claim 7, wherein said neurodegenerative disorder is Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer's disease, stroke, or epilepsy.

10

11. A method for treating or preventing the onset of a neurodegenerative disorder in a mammal, said method comprising administering to said mammal a therapeutically effective amount of a compound that decreases a neuronal cAMP level.

15

12. A method for identifying a mammal having or likely to develop a neurodegenerative disorder, said method comprising determining whether said mammal has an increased level of cellular cAMP in a neuron, said increased level indicating that said mammal has or is likely to develop a neurodegenerative disorder.

20

13. A method for identifying a mammal having or likely to develop a neurodegenerative disorder, said method comprising determining whether said mammal has a mutation in a cAMP regulatory gene, said mutation being an indication that said mammal has or is likely to develop a neurodegenerative disorder.

25

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14. The method of claim 13, wherein said mutation is in an adenylyl cyclase gene.

15. The method of claim 14, wherein said adenylyl cyclase gene is
5 the *acy-1* gene.

16. The method of claim 13, wherein said mutation is in the *unc-36* gene, said mutation is in the *eat-4* gene, or said mutation is in a gene encoding a $G\alpha_s$ subunit.

10

17. The method of claim 13, wherein said mutation results in an increase in a neuronal cAMP level.

18. The method of claim 11, 12, or 13, wherein said mammal is a
15 human.

19. The method of claim 18, wherein said neurodegenerative disorder is Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer's disease, stroke, or epilepsy.

20

20. A method for identifying a gene involved in neurodegeneration, said method comprising the steps of:

a) providing a nematode comprising an expression construct, said expression construct comprising a promoter derived from a cAMP regulatory
25 gene operably linked to a reporter gene;

b) isolating a mutant of said nematode exhibiting an altered level of reporter gene expression; and

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c) identifying said gene comprising said mutation, said gene being involved in neurodegeneration.

21. A method for identifying a gene involved in neurodegeneration,
5 said method comprising the steps of:

a) providing a nematode comprising a glutamate receptor (GluR) promoter operably linked to a gene encoding a GTP-ase defective $G\alpha_s$ subunit;

b) isolating a mutant of said nematode exhibiting a decreased level of paralysis and neurodegeneration; and

10 c) identifying said gene comprising said mutation, said gene being involved in neurodegeneration.

22. The method of claim 20 or 21, wherein said nematode is *C. elegans*.

15

23. A method for identifying a gene involved in neurodegeneration, said method comprising the steps of:

(a) providing a cell comprising a cAMP regulatory gene promoter operably linked to a reporter gene;

20 (b) introducing into said cell a candidate gene capable of expressing a candidate protein; and

(c) measuring reporter gene expression in said cell, an increase in said reporter gene expression in the presence of said candidate protein indicating that said candidate gene is involved in neurodegeneration.

25

24. The method of claim 23, wherein said cAMP regulatory gene is an *acy-1* gene, an *eat-4* gene, an *unc-36* gene, or a glutamate receptor-

-51-

encoding gene.

25. A mammalian EAT-4 polypeptide.

5 26. A purified nucleic acid encoding the polypeptide of claim 25.

27. The nucleic acid of claim 26, wherein said mammal is a human.

10 28. A vector comprising the nucleic acid of claim 26, said vector
being capable of directing expression of the said polypeptide in a vector-
containing cell.

29. A cell that contains the nucleic acid of claim 26.

I/II

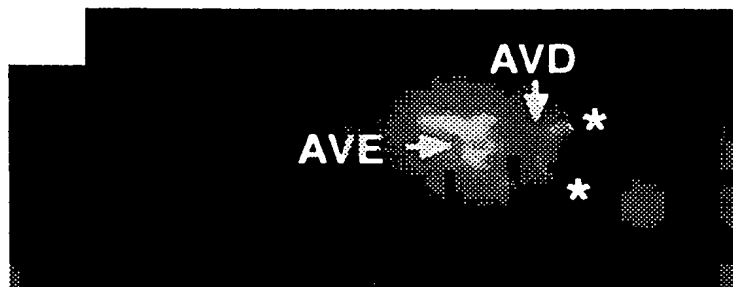


Fig. 1A



Fig. 1B

SUBSTITUTE SHEET (RULE 26)

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Role of cAMP and neural activity in G_s-induced neurotoxicity.

Swelling and cytotoxicity caused by the $\alpha_s(gf)$ transgene were quantitated in various genetic backgrounds, as described (9). For each data point 30-80 animals were analyzed.

<i>mut; $\alpha_s(gf)$ genotype</i> (MUT gene product)	% PVC Swelling	% PVC Degeneration
+	88	89

Adenylyl cyclase:

<i>acy-1 (nu327)</i>	19*	4*
<i>acy-1 (nu327)/+</i>	63*	ND
<i>acy-1 (nu329)</i>	0*	0*
<i>acy-1 (nu329)/+</i>	27*	ND
<i>acy-1 (nu343)</i>	4*	0*
<i>acy-1 (nu343)/+</i>	24*	ND

Degeneration:

<i>deg-1(u506u550) (ENaC)</i>	83	97
<i>mec-6(e1342)</i>	84	91
<i>unc-8(n491n1192) (ENaC)</i>	91	90

Calcium Channels:

<i>egl-19(n582) ($\alpha 2$ subunit)</i>	90	92
<i>unc-2(e55) ($\alpha 1$ subunit)</i>	86	82
<i>unc-36(e251) ($\alpha 1$ subunit)</i>	79	68*

Glutamate signaling:

<i>glr-1(n2461) (GluR A)</i>	82	95
<i>eat-4(ky5)</i>	78	58*

Apoptosis:

<i>ced-3(n717) (ICE)</i>	94	85
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Exocytosis:

<i>unc-18(e81) (n-Sec1)</i>	68* ¹	92 [‡]
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*Indicates significantly ($p < 0.005$) differs from $\alpha_s(gf)$ single mutants. ¹In addition to the swollen cells, 13% of PVC neurons in *unc-18* L1 larvae have condensed morphology characteristic of programmed cell deaths. [‡]25% of PVC corpses in *unc-18* adults appear to be engulfed by surrounding hypodermal cells.

Fig. 2

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MSSWNEAWDRGKQMVGEPLAKMTAAAASATGAAPPQQMQEEGNENPMQMH
SNKVLQVMEQTWIGKCRKRWLLAILANMGFMISFGIRCNFGAACKTHMYKN
YTDPYGKVMHEFNWTIDELSVMESSYFYGYLVTQIPAGFLAAKFPPNKL
FGFGIGVGAFNLNLLPYGFKVKS DYLVAFIQITQGLVQGVCPAMHGVWR
YWAPPMERSKLATTAFTGSYAGAVLGLPLSAFLVSVSWAAPFYLYGVCG
VIWAILWFCVTFEKPAFHPTISQEEKIFIEDAIGHVSNTHPTIRSIPWKA
IVTSKPVWAIIVANFARSWTFYLLLQNQLTYMKEALGMKIADSGLLAAIP
HLVMGCVVLMGGQLADYLR SNKILSTTAVRKIFNCGGFGGEAAFMLIVAY
TTSDTTAIMALIAAVGMSGFAISGFNVNHLDIAPRYAAILMGFSNGIGTL
AGLTCPFVTEAFTAHSKHGWT SVFLLASLIHFTGVTFYAVYASGELQEWA
EPKEEEEWSNKELVNKTGINGTGYGAAETTF TQLPAGVDSSYQAQAAPAP
GTNPFASAWDEHGSSGVVENPHYQQW

Fig. 3

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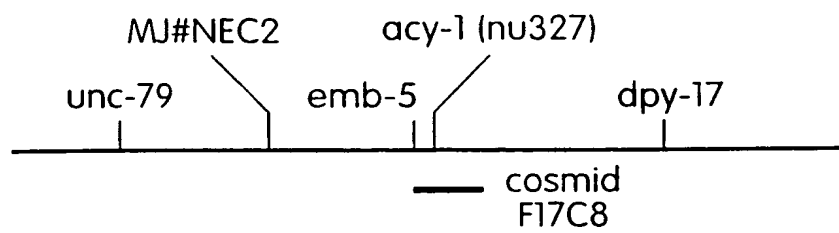


Fig. 4

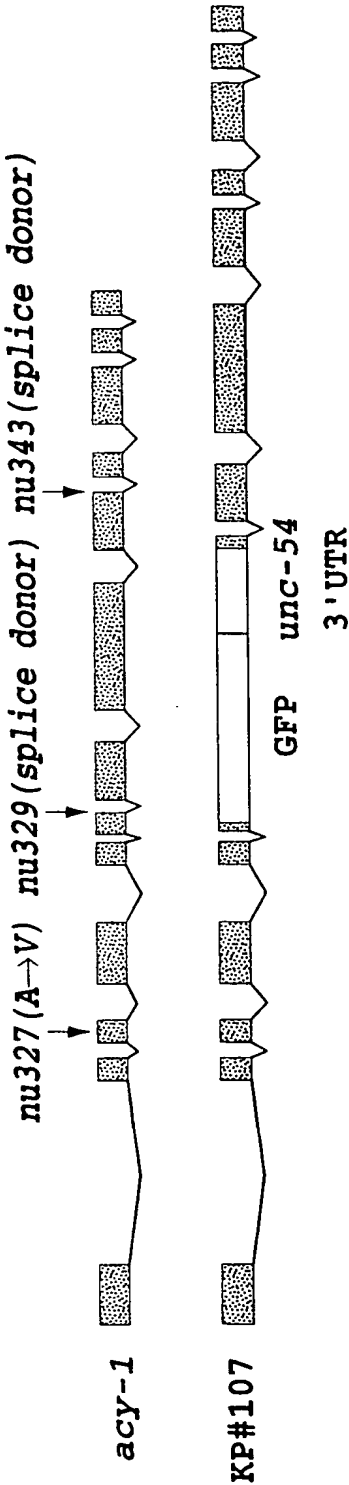


Fig. 5

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Fig. 6 (page 1 of 2)

SD nu343

Fig. 6 (page 2 of 2)

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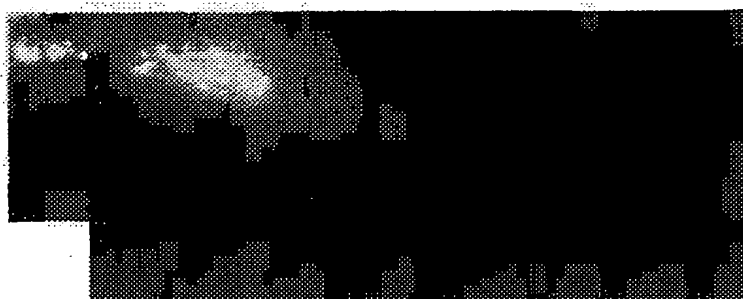


Fig. 7A

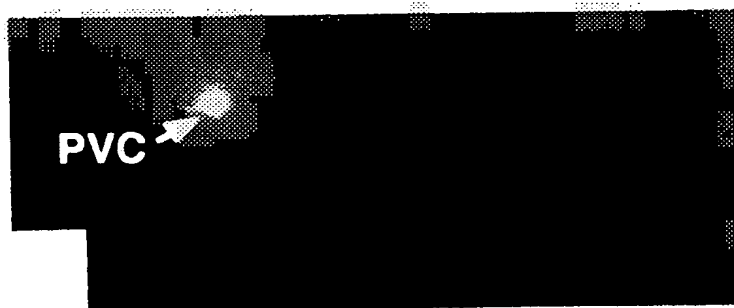


Fig. 7B

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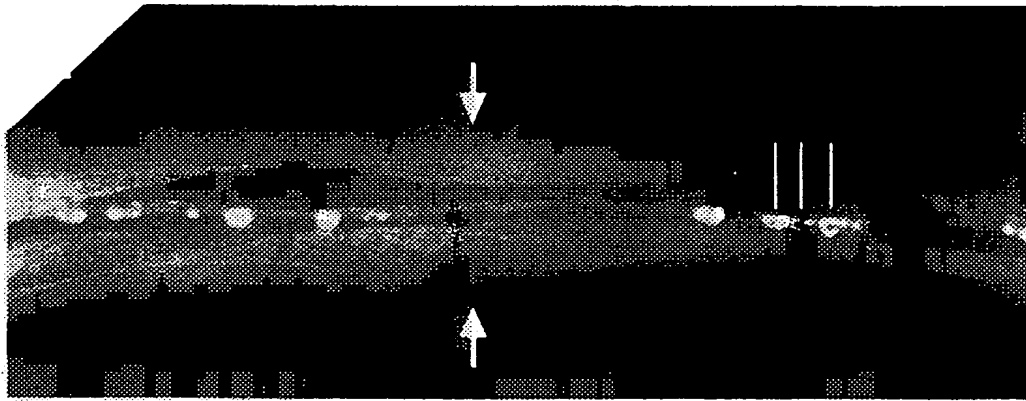


Fig. 8A

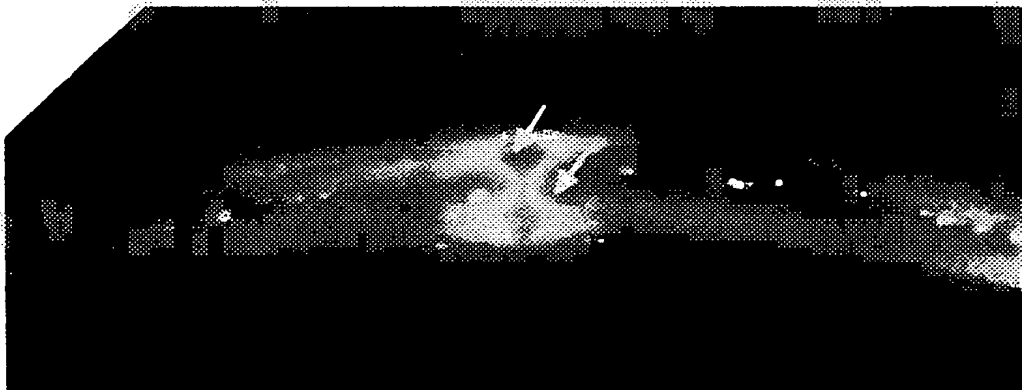


Fig. 8B

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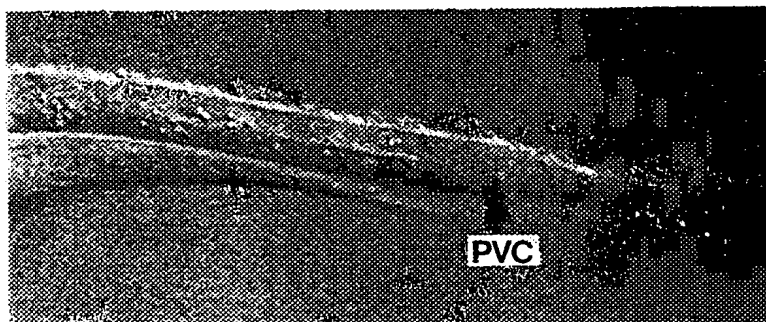


Fig. 9A

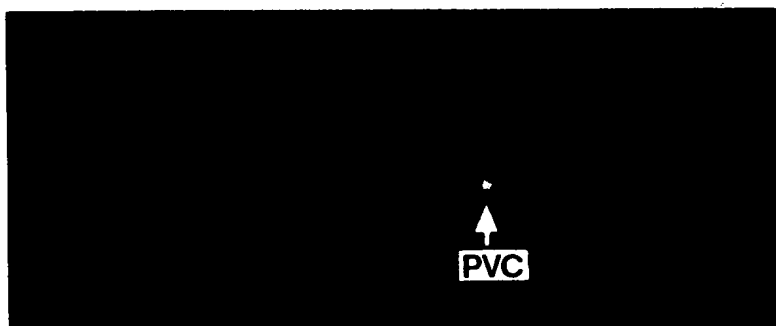


Fig. 9B

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Fig. 10A

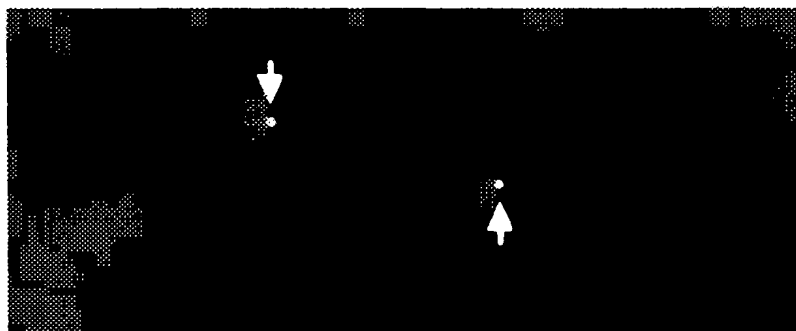


Fig. 10B

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11058

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9.1; 435/6, 172.3, 320.1, 325; 514/2, 44, 903; 530/350; 536/23.5; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, SCISEARCH, EMBASE, BIOSIS, CAPLUS, BIOTECHDS, DISSABS, CONFSCI, LIFESCI
elegans, glutam?, receptor#, neurodegen?, cyclase#

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SVENSSON et al. Heterologous Expression of the Cloned Guinea Pig α_2A , α_2B , and α_2C Adrenoceptor Subtypes. Biochem. Pharmacol. 09 February 1996, Volume 51, pages 291-300, especially pages 291 and 298-300.	1,3,6,7,10
A	WALDMANN et al. Functional Degenerin-containing Chimeras Identify Residues Essential for Amiloride-sensitive Na^+ Channel Function. J. Biolog. Chem. 19 May 1995, Volume 270, Number 20, pages 11735-11737.	1-3,6,7,10



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

01 SEPTEMBER 1998

Date of mailing of the international search report

13 OCT 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

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Authorized officer

STEPHEN GUCKER

Telephone No. (703) 308-0196



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11058**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3, 6-7, 10

Remark on Protest

☐
☐

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11058

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 49/00; C12N 5/00, 5/06, 5/10, 5/16, 15/00, 15/01, 15/09, 15/11, 15/12

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/9.1; 435/6, 172.3, 320.1, 325; 514/2, 44, 903; 530/350; 536/23.5; 800/2

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-3, 6-7, and 10, drawn to a method for identifying compounds limited to using cells comprising the *acy-1* gene and the cells themselves.

Group II, claim(s) 1-3, 6-7, and 10, drawn to a method for identifying compounds limited to using cells comprising the *eat-4* gene, and the cells themselves.

Group III, claim(s) 1-3, 6-7, and 10, drawn to a method for identifying compounds limited to using cells comprising the *unc-36* gene, and the cells themselves.

Group IV, claim(s) 1-3, 6-7, and 10, drawn to a method for identifying compounds limited to using cells comprising the glutamate receptor-encoding gene, and the cells themselves.

Group V, claim(s) 1-6 and 8-10, drawn to a method for identifying compounds limited to using an animal comprising the *acy-1* gene.

Group VI, claim(s) 1-6 and 8-10, drawn to a method for identifying compounds limited to using an animal comprising the *eat-4* gene.

Group VII, claim(s) 1-6 and 8-10, drawn to a method for identifying compounds limited to using an animal comprising the *unc-36* gene.

Group VIII, claim(s) 1-6 and 8-10, drawn to a method for identifying compounds limited to using an animal comprising the glutamate receptor-encoding gene.

Group IX, claim(s) 11 and 18-19, drawn to a method for treating a neurodegenerative disorder in a mammal by administering a compound to decrease neuronal cAMP level.

Group X, claim(s) 12 and 18-19, drawn to a method for identifying a mammal having or likely to develop a neurodegenerative disorder by determining an increased level of cellular cAMP in a neuron.

Group XI, claim(s) 13-15 and 17-19, drawn to a method for identifying a mammal having or likely to develop a neurodegenerative disorder by determining a mutation in a cAMP regulatory gene limited to an adenylyl cyclase gene.

Group XII, claim(s) 13-14 and 16-19, drawn to a method for identifying a mammal having or likely to develop a neurodegenerative disorder by determining a mutation limited to the *unc-36* gene.

Group XIII, claim(s) 13-14 and 16-19, drawn to a method for identifying a mammal having or likely to develop a neurodegenerative disorder by determining a mutation limited to the *eat-4* gene.

Group XIV, claim(s) 13-14 and 16-19, drawn to a method for identifying a mammal having or likely to develop a neurodegenerative disorder by determining a mutation limited to the a gene encoding a G-alpha-s subunit.

Group XV, claims(s) 20-22, drawn to a method for identifying genes involved in neurodegeneration in a nematode.

Group XVI, claim(s) 23-24, drawn to a method for identifying genes involved in neurodegeneration by using cAMP regulatory gene promoters linked to reporter genes.

Group XVII, claim(s) 25, drawn to mammalian EAT-4 polypeptide.

Group XVIII, claim(s) 26-29, drawn to purified nucleic acid, a vector, and host cell.

The inventions listed as Groups I-XVIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature linking groups I-XVIII appears to be that they all relate to an identifying method or product identified or used by a method involving measuring or altering the level or activity of cellular cAMP either directly or by reporter or regulatory gene expression.

However, Svensson et al. (1996) teaches methods to identify and products so identified (agonists and antagonists) that alter the expression of the cAMP-responsive reporter gene chloramphenicol acetyltransferase (CAT) (see abstract and

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/11058

pages 298-299). The teachings of Svensson et al. meet all the limitations of claim 1 and the compounds identified on Table I could be used to lower the level of cAMP as shown in Figure 6 to treat a neurodegenerative disorder.

Therefore, the technical feature linking the inventions of Groups I-XVIII does not constitute a special technical feature as defined by PCT Rule 13.2, as it does not define a contribution over the prior art.